Remarks

Claims 1-8 were pending in the subject application. By this Amendment, the applicants have amended claims 1, 2 and 6, added claims 22-34 and cancelled claims 9-21. No new matter has been added by these amendments. Support for the amendments can be found throughout the subject specification and claims as originally filed (see, for example, the paragraph bridging pages 5-6, page 6, paragraph 3 and page 12, paragraph 1). Accordingly, claims 1-8 and 22-34 are currently before the Examiner. Favorable consideration of the pending claims, in view of the amendments and remarks set forth herein, is earnestly solicited.

The claim amendments set forth herein have been done in order to lend greater clarity to the claimed subject matter and to expedite prosecution. The amendments should not be taken to indicate the applicants' agreement with, or acquiescence to, the rejections of record. Favorable consideration of the claims now presented, in view of the remarks and amendments set forth herein, is respectfully requested.

Claims 1-8 have been rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. The Office Action argues that the as-filed specification fails to describe the physical or chemical properties of the claimed class of proteins, chelating agents, coupling reagents or enzymes recited in the claims and that the skilled artisan would not have recognized that the applicants were in possession of the claimed invention. For example, where the Office Action alleges that the specification fails to describe "in any fashion" the physical and/or chemical properties of the claimed class of: a) the proteins to be analyzed; b) the claimed chelating agents ("Y"); c) the chemical coupling agent ("PRG" and "A"), and d) enzyme used in cleavage. Applicants respectfully disagree with the position of the Examiner and traverse.

As indicated in *Capon v. Eshhar*, 418 F.3d 1349, 1357, 76 U.S.P.Q.2d 1078, 1084 (Fed. Cir. 2005),

> The "written description" requirement implements the principle that a patent must describe the technology that is sought to be patented; the requirement serves both to satisfy the inventor's obligation to disclose the technologic knowledge upon which the patent is based, and to demonstrate that the patentee was in possession of the invention that is claimed. *See Enzo Biochem*, 296 F.3d at 1330 (the written description requirement "is the quid pro quo of the patent system; the public must

receive meaningful disclosure in exchange for being excluded from practicing the invention for a limited period of time"); *Reiffin v. Microsoft Corp.*, 214 F.3d 1342, 1345-46 (Fed. Cir. 2000) (the purpose of the written description requirement "is to ensure that the scope of the right to exclude . . . does not overreach the scope of the inventor's contribution to the field of art as described in the patent specification"); *In re Barker*, 559 F.2d 588, 592 n.4 (CCPA 1977) (the goal of the written description requirement is "to clearly convey the information that an applicant has invented the subject matter which is claimed"). The written description requirement thus satisfies the policy premises of the law, whereby the inventor's technical/scientific advance is added to the body of knowledge, as consideration for the grant of patent exclusivity.

The descriptive text needed to meet these requirements varies with the nature and scope of the invention at issue, and with the scientific and technologic knowledge already in existence. The law must be applied to each invention that enters the patent process, for each patented advance is novel in relation to the state of the science. Since the law is applied to each invention in view of the state of relevant knowledge, its application will vary with differences in the state of knowledge in the field and differences in the predictability of the science.

Additionally, "[a] patent need not teach, and preferably omits, what is well known in the art." *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 1534 (Fed.Cir.1987)", see *Falko-Gunter Falkner v. Inglis*, 448 F.3d 1357, 1365, 79 U.S.P.Q.2d 1001 (Fed. Cir. 2006)). In the case of the claimed invention, it is respectfully submitted that the application of the law, in view of the state of the art, should result in a finding that the specification reasonably conveys to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention.

The method according to the invention comprises the steps of: a) providing a sample, which contains a mixture of proteins. By definition, initially the proteins are present in the sample and their amounts are unknown (and actually clarifying this is the focus of the present claims). Nevertheless, it is known that they are derived from the proteome of a cell. The next step of the claimed method provides a reagent for the analysis of peptides, which has the general formula A-Y-PRG, in which A constitutes at least one functional group for the reversible, covalent or non-covalent binding to a support material; in which Y is a group comprising at least one chelate function for metals and comprising a metal ion bound thereto, wherein the metal is selected from the group consisting of Ag, Al, As, Au, Be, Cd, Ce, Co, Cr, Cu, Dy, Er, Eu, Fe, Gd, Hg, Ho, In, La, Li, Lu, Mn, Na, Nd, Ni, Pb, Pr, Rb, Rd, Sb, Sm, Sn, Tb, Tl, Tm, V, W, Y, Yb and Zn; and in which PRG is a reactive group for

the selective binding to peptides or other biomolecules to be analyzed. Thus, steps a) and b) define how the analysis of the protein mixture is to be started and then the following steps are performed: c) cleaving the proteins in the sample in order to produce peptides; d) coupling the peptides to the reagent of step b); e) selecting the peptides labeled in step d) by the employment of reversible binding to a support material or of affinity labeling by the binding to a support material and the removal of unbound peptides; f) releasing the bound peptides from the support material and elution from the matrix; and g) detecting und identifying the labeled peptides by means of mass spectrometry.

As noted above, the present invention relates to a method for a reproducible, systematic, qualitative and quantitative characterization of the proteome by means of non-isotope metal coded markers and tandem methods of mass spectrometry (specification, page 1, introduction). According to the specification, the proteome consists of all proteins being expressed within a cell or tissue. The specification, thus, teaches that the analysis of the proteome requires the identification of one or more protein (as part(s) of the proteome). Thus, in the context of this application, the class of proteins are all those proteins produced by a cell or tissue being analyzed by the claimed method. Applicants further note that the Examples discuss a test mixture of 5-10 proteins and a number of "real life samples" were tested (Example I, "Concept of the experiment" 3.)

Turning to the issue of chelators, the written description requirement should not be so burdensome as to prohibit applicant from claiming "undisclosed but obviously art-recognized equivalents" of expressly disclosed aspects of the invention (In re Smythe, 480 F.2d 1376, 1384 (CCPA 1973). Additionally, "[a] patent need not teach, and preferably omits, what is well known in the art." *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 1534 (Fed.Cir.1987)", see *Falko-Gunter Falkner v. Inglis*, 448 F.3d 1357, 1365, 79 U.S.P.Q.2d 1001 (Fed. Cir. 2006)). In this case, the specification states at page 10: "An essential function of the reagent according to the present invention is its chelate forming function. In preferred reagents according to the present invention, Y is selected from a macrocyclic lanthanoid chelate complex, a functionalized tetraaza-macrocycle, a polyaza-polyacetic acid, DOTA, a DOTA-derivative, NOTA, a NOTA-derivative, EDTA, DTPA-BP, DTPA, DO3A, HP-DO3A and DTPA-BMA. Particularly preferred compounds are 1,4,7,10,13,16,19,22-octaazacyclotetracosane-1,4,7,10,13,16,19,22-octaacetic acid (OTEC), and

1,4,7,10,14-17,20,23-octaazacyclohexacosane-1,4,7,10,14,17,20,23-octaacetic acid (OHEC)", and further "The metals, which can be bound by the chelate-forming function of the reagent according to the present invention, can be selected from a large variety of metals, thereby significantly improving the flexibility when using the reagent according to the present invention. Thus, the metal bound by the chelate complex can be selected from Ag, Al, As, Au, Be, Cd, Ce, Co, Cr, Cu, Dy, Er, Eu, Fe, Gd, Hg, Ho, In, La, Li, Lu, Mn, Na, Nd, Ni, Pb, Pr, Rb, Rd, Sb, Sm, Sn, Tb, Tl, Tm, V, W, Y, Yb and Zn. According to the invention, the chelate-forming group can be labeled with several different metals." Additionally, a wide variety of chelators are known to those skilled in the art and commercially available from vendors, such as the Sigma Aldrich Co. (see attached printout). Thus, Applicant is of the opinion that there is sufficient written description in order to show broad possession of the chelate forming groups, at least macrocyclic ligands, and respective metals.

With respect to the functional linker groups associated with the general formula provided in the independent claims, the specification states (at page 9, 2nd paragraph): "Preferably, the function PRG is selected from a sulfhydryl-reactive group, an amine-reactive group and an enzyme substrate. It is moreover preferred, that PRG is selected from the group of an amine-reactive pentafluorophenyl ester group, an amine-reactive N-hydroxysuccinimide ester group, sulfonylhalide, isocyanate, isothiocyanate, active ester, tetrafluorophenyl ester, an acid halide and an acid anhydride, a homoserine lactone-reactive primary amine group and a carboxylic acid-reactive amine, alcohol or 2,3,5,6-tetrafluorophenyltrifluoro-acetate, a iodine acetylamide group, an epoxide, an α-haloacyl group, a nitrile, a sulfonated alkyl, an arylthiol and a maleimide." Furthermore, regarding "A" the specification on page 9, in paragraphs 4 and 5, further states: "Particularly preferred is a reagent according to the invention, in which A is selected from biotin or modified biotin, a 1,2-diol, glutathione, maltose, a nitrilotriacetic acid group, an oligohistidine or a hapten. In case of biotin, the reagent can *e.g.* be coupled to a streptavidin group in order to allow its convenient isolation. Particularly preferred in this context is the employment of a streptavidin-labeled column matrix or of coated beads", and "in a further embodiment, A is a reactive group coupled to the support material, which reactive group can again be cleaved off the support material. Possible options for this are - among other things - disulfide bonds (S-S), which can be reduced again, thus leading to cleavage, or photosensitive bonds, which can be cleaved by exposure to light.

Applicants also note that the specification discloses that proteins can be labeled at specific binding sites, such as sulfhydryl groups, thus exclusively binding to proteins containing cysteine. Subsequently, only the cysteine-containing peptides are isolated in a selective manner, and analyzed further (see WO 00/11208). It is also respectfully submitted that the person of skill in the art is also aware of other ways to label proteins and the various reaction chemistries used to label the proteins with linker demands, such as biotinylation (see, for example, piercenet.com/Proteomics/browse.cfm?fldID=84EBE112-F871-4CA5-807F-47327153CFCB or the attached material from the Pierce Chemical Company (Crosslinking Reagent Technical Handbook; Appendix I).

Finally, the specification on page 7, in paragraph 1 states: "Preferred is a method according to the invention, in which the cleavage is accomplished in an enzymatic or chemical way. The cleavage can be appropriately performed by a hydrolysis under the employment of known proteases like *e.g.* trypsin, ASP-N-protease, pepsin, Lys-C, Glu-C, Arg-C proteinase, Asp-N endopeptidase, BNPS-scatoles, caspases, chymotrypsin, clostripain, factor Xa, glutamyl-endopeptidase, granzyme B, proline endopeptidase, proteinase K, staphylococcus peptidase A, thermolysin, thrombin, carboxypeptidases and combinations thereof. The chemical cleavage can be performed by means of partial acid hydrolysis, CNBr, formic acid, iodosobenzoic acid, NTCB (2-nitro-5-thiocyano benzoic acid), hydroxylamine, and combinations thereof." Furthermore, various chemicals and enzymes capable of cleaving proteins (as well as their respective cleavage specificities) were known to those skilled in the art at the time the invention was made (see, for example, the attached printout from EMBL). Thus, it is clear that the as-filed specification provides adequate written description for the enzymes that are used for cleavage and those skilled in the art would also be able to select an enzyme for cleavage of a target based upon the bonds to be hydrolyzed. Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention is respectfully requested.

Claims 1-8 have been rejected under 35 U.S.C. §112, first paragraph as being non-enabled. The Office Action argues that the as-field specification fails to enable one skilled in the art to

identify and quantify a genus of proteins using: any chelating agent, any chemical compound bound to a solid support, and any proteolytic enzyme as claimed followed by the use of any HPLC/MS methodology. The Office Action further argues that the specification does not provide sufficient guidance to enable one skilled in the art to make and use the claimed invention in a manner reasonably correlated with the scope of the claims. The applicants respectfully traverse this ground for rejection because the person of ordinary skill in the art could readily, and without undue experimentation, practice the full scope of the invention as set forth in the claims that are now presented for examination.

As noted above, the claimed method is directed to a reproducible, systematic, qualitative and quantitative characterization of the <u>proteome</u> by means of non-isotope metal coded markers and tandem methods of mass spectrometry (specification, page 1, introduction). According to the specification, the <u>proteome</u> consists of all proteins being expressed within a cell or tissue.

Enablement is a legal determination of whether a patent enables one skilled in the art to make and use the claimed invention (*Raytheon Co. v. Roper Corp.*, 724 F.2d 951, 960, 220 U.S.P.Q. 592, 599 (Fed. Cir. 1983)) and is not precluded even if some experimentation is necessary. *Atlas Powder Co. v. E.I. Du Pont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 U.S.P.Q. 409, 413 (Fed. Cir. 1984); *W.L. Gore and Associates v. Garlock, Inc.*, 721 F.2d 1540, 1556, 220 U.S.P.Q. 303, 315 (Fed. Cir. 1983). Applicants also submit that nothing more than objective enablement is required, and therefore, it is irrelevant whether this teaching is provided through broad terminology or illustrative examples. Additionally, the Patent and Trademark Office Board of Patent Appeals and Interferences has stated: "The test [for enablement] is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed". *Ex parte Jackson*, 217 U.S.P.Q. 804, 807 (1982); *see also Ex parte Erlich* 3 U.S.P.Q.2d 1011 (B.P.A.I. 1982). Applicants further submit that the degree of guidance argued to be necessary in the Office Action is not required to meet the enablement requirement of 35 U.S.C. § 112. *See In re Chilowsky*, 229 F.2d 457, 460, 108 U.S.P.Q. 321, 324 (CCPA 1956) ("[T]he applicant 'may begin at the point where his invention begins, and describe what he has made that is new and what it replaces of the old. That which is common and well known is as if it were written out in the patent and

delineated in the drawings.'"); *Hybritech, Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 U.S.P.Q. 81, 94 (Fed. Cir. 1987).

With this legal background in mind, Applicants respectfully submit that the as-filed specification is enabled in view of the knowledge and information available to those skilled in the art and the teachings of the as-filed specification. For example, various chelators, the chemical structures of the chelators and the metals each chelators chelates would have been known to those skilled in the art at the time the invention was made (see, for example, page 10 and the attached Sigma Aldrich printout identifying known chelators). Likewise, a number of chemical linkers, their chemical structure and the reaction chemistry of the chemical linkers was known to those skilled in the art at the time this application was filed (see, for example, piercenet.com/Proteomics/browse.cfm?fldID=84EBE112-F871-4CA5-807F-47327153CFCB or the attached material from the Pierce Chemical Company (Crosslinking Reagent Technical Handbook; Appendix I). Likewise, chemicals and various enzymes capable of cleaving proteins (as well as their respective cleavage specificities) were known to those skilled in the art at the time the invention was made (see, for example, page 7 and the attached printout from EMBL). Accordingly, it is respectfully submitted that the as-filed specification fully enables the claimed invention and reconsideration and withdrawal of the rejection is respectfully requested.

Claims 1-8 have been rejected under 35 U.S.C. §102(b) as being anticipated by Aebersold *et al.* (WO 00/11208). The applicants respectfully traverse this ground for rejection because the cited reference does not disclose each and every element of the claimed invention. WO 00/11208 discloses the isotope-coded affinity tag (ICAT) method for proteome analysis, using isotope-coded affinity tags (ICATs) and methods of mass spectrometry. The proteins, after being isolated from the cells, are labeled by the ICAT-reagent at specific binding sites, such as sulfhydryl groups, thus exclusively binding to proteins containing cysteine. Subsequently, only the cysteine-containing peptides are isolated in a selective manner, and analyzed further. Instead of an <u>isotope</u> labeling, the present invention provides the integration of a metal <u>ion</u> chelate complex into the reagent. Thus, it is respectfully submitted that the claims are novel over Aebersold et al. (WO 00/11208) and reconsideration and withdrawal of the rejection is respectfully requested.

In view of the foregoing remarks and the amendment above, the applicants believe that the currently pending claims are in condition for allowance, and such action is respectfully requested.

The Commissioner is hereby authorized to charge any fees under 37 CFR §§1.16 or 1.17 as required by this paper to Deposit Account No. 19-0065.

The applicants also invite the Examiner to call the undersigned if clarification is needed on any of this response, or if the Examiner believes a telephone interview would expedite the prosecution of the subject application to completion.

Respectfully submitted,

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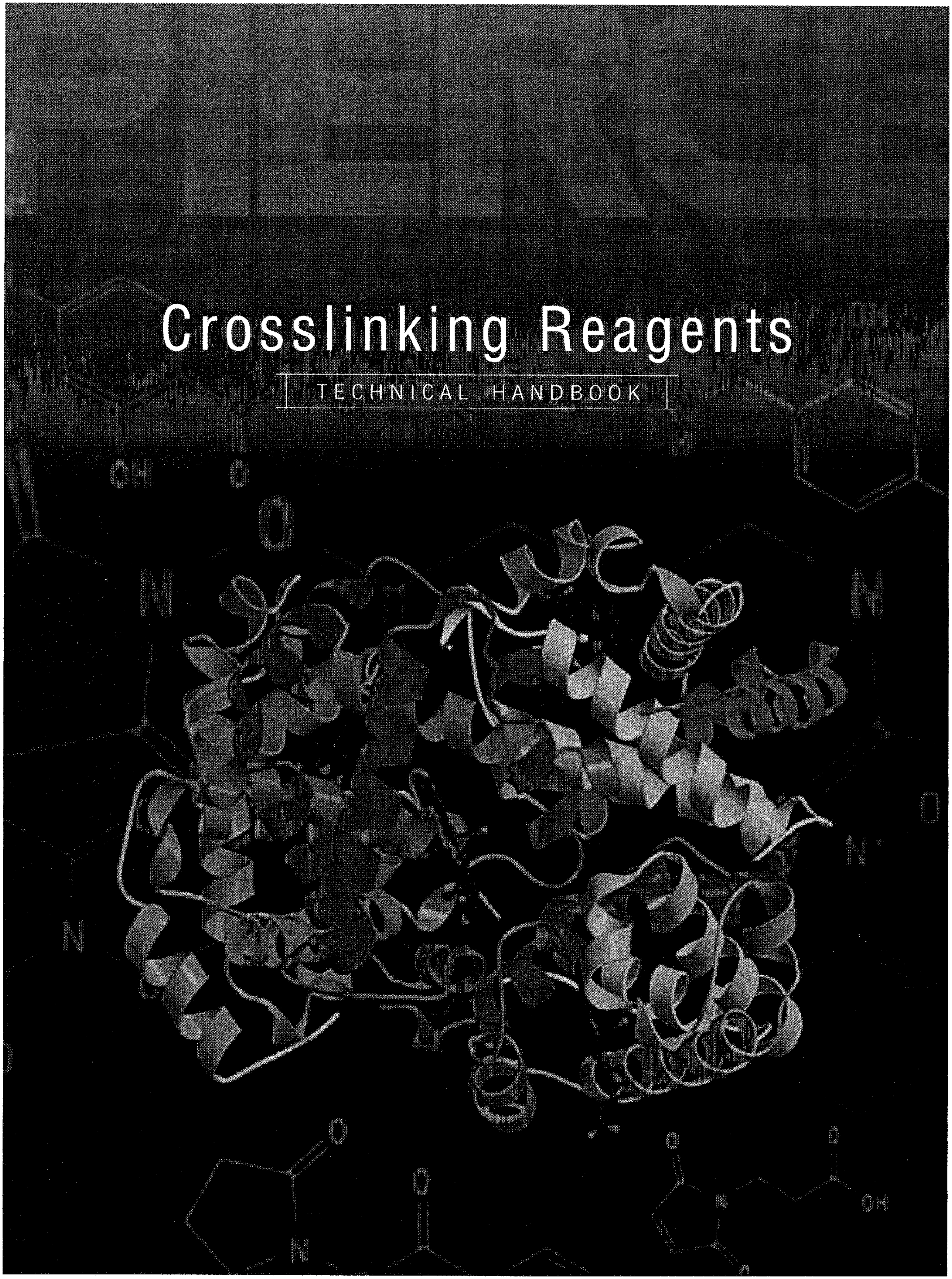
FCE/jb

Attachments: Chelators, BioUltra Reagents, Sigma Aldrich Co. printout
piercenet.com/Proteomics/browse.cfm?fldID=84EBE112-F871-4CA5-807F-47327153CFCB
Crosslinking Reagent Technical Handbook
EMBL printout (Peptide Cutter)

Crosslinking Reagents

TECHNICAL HANDBOOK



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Introduction to crosslinking

What is crosslinking?

Crosslinking is the process of chemically joining two or more molecules by a covalent bond. Crosslinking reagents contain reactive ends to specific functional groups (primary amines, sulfhydryls, etc.) on proteins or other molecules. Because of the availability of several chemical groups in proteins and peptides that may be targets for reactions, proteins and peptides are readily conjugated and otherwise studied using crosslinking methods. Crosslinkers also are commonly used to modify nucleic acids, drugs and solid surfaces. Crosslinking reagents have been used to assist in determination of near-neighbor relationships, three-dimensional structures of proteins, solid-phase immobilization, hapten-carrier protein conjugation and molecular associations in cell membranes. They also are useful for preparing antibody-enzyme conjugates, immunotoxins and other labeled protein reagents.

Conformational changes of proteins associated with a particular interaction may be analyzed by performing crosslinking studies before and after the interaction occurs. Comparing crosslinkers with different arm lengths for success of conjugation can provide information about the distances between interacting molecules. By examining which crosslinkers effectively conjugate to particular domains of a protein, information may be obtained about conformational changes that hindered or exposed amino acids in the tertiary and quaternary structure.

The use of crosslinkers has made the study of surface receptors much easier. By derivatizing a receptor with a crosslinker before or after contact with the ligand, it is possible to isolate the receptor-ligand complex. The use of radioiodinatable crosslinkers makes it possible to identify a particular receptor by autoradiographic detection.

How to choose a crosslinker

Crosslinkers are selected on the basis of their chemical reactivities (i.e., specificity for particular functional groups) and compatibility of the reaction with the application. The best crosslinker to use for a specific application must be determined empirically. Crosslinkers are chosen based on the following characteristics:

- Chemical specificity
- Spacer arm length
- Water solubility and cell membrane permeability
- Same (homobifunctional) or different (heterobifunctional) reactive groups
- Spontaneously reactive or photoreactive groups
- Cleavability
- Reagent contains moieties that can be radiolabeled or tagged with another label

Crosslinkers contain at least two reactive groups. Functional groups that can be targeted for crosslinking include primary amines, sulfhydryls, carbonyls, carbohydrates and carboxylic acids (Table 1). Coupling also can be nonselective using a photoreactive phenyl azide crosslinker. The Pierce web site (*www.piercenet.com*) contains a crosslinker selection guide by which the above-listed parameters may be chosen and a list of available crosslinkers with those features generated.

Table 1. Reactive crosslinker groups and their functional group targets.

Reactive Group	Target Functional Group	Reactive Group	Target Functional Group
Aryl Azide	Nonselective (or primary amine)	Maleimide	Sulfhydryl
Carbodiimide	Amine/Carboxyl	NHS-ester	Amine
Hydrazide	Carbohydrate (oxidized)	PFP-ester	Amine
Hydroxymethyl Phosphine	Amine	Psoralen	Thymine (photoreactive intercalator)
Imidoester	Amine	Pyridyl Disulfide	Sulfhydryl
Isocyanate	Hydroxyl (non-aqueous)	Vinyl Sulfone	Sulfhydryl, amine, hydroxyl
Carbonyl	Hydrazine	Carbonyl	Hydrazine

How to choose a crosslinker (continued)

Often different spacer arm lengths are required because steric effects dictate the distance between potential reaction sites for crosslinking. For protein:protein interaction studies, the proximity between reactive groups is difficult to predict. Usually, a crosslinker with a short (4-8 Å) spacer arm is used first and the degree of crosslinking determined. A crosslinker with a longer spacer arm may then be used to optimize crosslinking efficiency. Short spacer arms are often used in intramolecular crosslinking studies, and intermolecular crosslinking is favored with a crosslinker containing a long spacer arm. Often crosslinkers that are cleavable, non-cleavable and have various spacer arm lengths are used to obtain a complete analysis of protein structure.

General reaction conditions

In many applications, it is necessary to maintain the native structure of the protein complex, so crosslinking is most often performed using mild pH and buffer conditions. Furthermore, optimal crosslinker-to-protein molar ratios for reactions must be determined. Depending on the application, the degree of conjugation is an important factor. For example, when preparing immunogen conjugates, a high degree of conjugation is desired to increase the immunogenicity of the antigen. However, when conjugating to an antibody or an enzyme, a low- to moderate-degree of conjugation may be optimal so that biological activity of the protein is retained. The number of functional groups on the protein's surface is also important to consider. If there are numerous target groups, a lower crosslinker-to-protein ratio can be used. For a limited number of potential targets, a higher crosslinker-to-protein ratio may be required. Furthermore, the number of components should be kept to a minimum because conjugates consisting of more than two components are difficult to analyze and provide less information on spatial arrangements of protein subunits.

Water solubility and membrane permeability

Many crosslinkers, by virtue of their hydrophobic spacer arms, have limited solubility in aqueous solutions. These crosslinkers are generally dissolved in DMF or DMSO, then added to the biological system or solution of biomolecules to be crosslinked. Hydrophobic crosslinkers are able to cross cellular and organellar membranes and effect crosslinking both at the outer surface of a membrane and within the membrane-bounded space.

It is often inconvenient or undesirable to introduce organic solvents into a crosslinking procedure for a biological system. It is also desirable in many instances to effect crosslinking only on the outer surface of a cellular or organellar membrane without altering the interior of the cell or organelle and, in such cases, several water-soluble, membrane-impermeable crosslinkers are available. Some crosslinkers contain a spacer arm formed from polyethylene glycol (PEG) subunits and resulting in a polyethylene oxide (PEO) chain with abundant oxygen atoms to provide water solubility. These crosslinkers are designated by a $(PEO)_n$ in their name and are both water-soluble and unable to penetrate biological membranes. They provide the added benefit of transferring their hydrophilic spacer to the crosslinked complex, thus decreasing the potential for aggregation and precipitation of the complex. Other crosslinkers obtain their water-solubility and membrane-impermeability by virtue of a charged reactive group at either end of the spacer. These charged reactive groups, such as sulfo-NHS esters or imidoesters, impart water-solubility to the crosslinking reagent, but not to the crosslinked complex because the reactive group is not a part of the final complex.

Crosslinking applications

Cell surface crosslinking

Crosslinkers are often used to identify surface receptors or their ligands. Membrane-impermeable crosslinkers ensure cell-surface-specific crosslinking. Water-insoluble crosslinkers when used at controlled amounts of reagent and reaction time can reduce membrane penetration and reaction with inner membrane proteins.

The sulfonyl groups attached to the succinimidyl rings of NHS-esters result in a crosslinker that is water-soluble, membrane-impermeable and nonreactive with inner-membrane proteins. Therefore, reaction time and quantity of crosslinker are less critical when using sulfo-NHS-esters. Homobifunctional sulfo-NHS-esters, heterobifunctional sulfo-NHS-esters and photoreactive phenyl azides are good choices for crosslinking proteins on the cell surface.

Determination of whether a particular protein is located on the surface or the integral part of the membrane can be achieved by performing a conjugation reaction of a cell membrane preparation to a known protein or radioactive label using a water-soluble or water-insoluble crosslinker. Upon conjugation the cells may be washed, solubilized and characterized by SDS-polyacrylamide gel electrophoresis (PAGE) to determine whether the protein of interest was conjugated. Integral membrane proteins will form a conjugate in the presence of a water-insoluble crosslinker, but not in the presence of water-soluble crosslinkers. Surface membrane proteins can conjugate in the presence of water-soluble and water-insoluble crosslinkers. BASED (Product # 21564), a homobifunctional photoactivatable phenyl azide, is one of the more versatile crosslinkers for the study of protein interactions and associations. It is cleavable and can be radiolabeled with ^{125}I using IODO-BEADS® Iodination Reagent (Product # 28665). After cleavage, both of the dissociated molecules will still be iodinated. Because both reactive groups on this crosslinker are nonspecific, the crosslinking is not dependent on amino acid composition for successful conjugation.

Cell membrane structural studies

Cell membrane structural studies require reagents of varying hydrophobicity to determine the location and the environment within a cell's lipid bilayer. Fluorescent tags are used to locate proteins, lipids or other molecules inside and outside the membrane. Various crosslinkers, with differing spacer arm lengths, can be used to crosslink proteins to associated molecules within the membrane to determine the distance between molecules. Successful crosslinking with shorter crosslinkers is a strong indication that two molecules are interacting in some manner. Failure to obtain crosslinking with a panel of shorter crosslinkers, while obtaining conjugation with the use of longer reagents, generally indicates that the molecules are located in the same part of the membrane, but are not interacting. Homobifunctional NHS-esters, imidates or heterobifunctional NHS-ester/photoactivatable phenyl azides are commonly used for these procedures. Although imidoester crosslinkers (imidates) are water-soluble, they are still able to penetrate membranes. Sulfhydryl-reactive crosslinkers may be useful for targeting molecules with cysteines to other molecules within the membrane.

EDC (Product # 22980, 22981), water-insoluble dicyclohexylcarbodiimide (DCC, Product # 20320) and other water-soluble/-insoluble coupling reagent pairs are used to study membranes and cellular structure, protein subunit structure and arrangement, enzyme:substrate interactions, and cell-surface and membrane receptors. The hydrophilic character of EDC can result in much different crosslinking patterns in membrane and subunit studies than with hydrophobic carbodiimides such as DCC. Often it is best to attempt crosslinking with a water-soluble and water-insoluble carbodiimide to obtain a complete picture of the spatial arrangements or protein:protein interactions involved.

Subunit crosslinking and protein structural studies

Crosslinkers can be used to study the structure and composition of proteins in samples. Some proteins are difficult to study because they exist in different conformations with varying pH or salt conditions. One way to avoid conformational changes is to crosslink subunits. Amine-, carboxyl- or sulfhydryl-reactive reagents are used for identification of particular amino acids or for determination of the number, location and size of subunits. Short- to medium-spacer arm crosslinkers are selected when intramolecular crosslinking is desired. If the spacer arm is too long, intermolecular crosslinking can occur. Carbodiimides that result in no spacer arm, along with short-length conjugating reagents, such as amine-reactive DFDNB (Product # 21525) or the photoactivatable amine-reactive crosslinker NHS-ASA (Product # 27714), can crosslink between subunits without crosslinking to extraneous molecules if used in optimal concentrations and conditions. Slightly longer crosslinkers, such as DMP (Product # 21666, 21667), can also crosslink between subunits, but they may result in intermolecular coupling. Adjusting the reagent amount and protein concentration can control intermolecular crosslinking. Dilute protein solutions and high concentrations of crosslinker favor intramolecular crosslinking when homobifunctional crosslinkers are used.

For determination or confirmation of the three-dimensional structure, cleavable crosslinkers with increasing spacer arm lengths may be used to determine the distance between subunits. Experiments using crosslinkers with different reactive groups may indicate the locations of specific amino acids. Once conjugated, the proteins are subjected to two-dimensional electrophoresis. In the first dimension, the proteins are separated using non-reducing conditions and the molecular weights are recorded. Some subunits may not be crosslinked and will separate according to their individual molecular weights. Conjugated subunits will separate according to the combined molecular weight. The second dimension of the gel is then performed using conditions to cleave the crosslinked subunits. The individual molecular weights of the crosslinked subunits can be determined. Crosslinked subunits that were not reduced will produce a diagonal pattern, but the cleaved subunits will be off the diagonal. The molecular weights of the individual subunits should be compared with predetermined molecular weights of the protein subunits using reducing SDS-polyacrylamide gel electrophoresis.

Protein interactions and associations

Crosslinkers are used for identification of near-neighbor protein relationships and ligand-receptor interactions. The crosslinkers chosen for these applications are usually longer than those used for subunit crosslinking. Homobifunctional, amine-reactive NHS-esters or imidates and heterobifunctional, amine-reactive, photoactivatable phenyl azides are the most commonly used crosslinkers for these applications. Occasionally, a sulfhydryl- and amine-reactive crosslinker, such as Sulfo-SMCC (Product # 22322), may be used if one of the two proteins or molecules is known to contain sulfhydryls. Both cleavable or noncleavable crosslinkers can be used. Because the distances between two molecules are not always known, the optimal length of the spacer arm of the crosslinker may be determined by the use of a panel of similar crosslinkers with different lengths. DSS (Product # 21555) or its cleavable analog DSP (Product # 22585) are among the shorter crosslinkers used for protein:protein interactions. NHS-ester, phenyl azides are very useful for this type of crosslinking because they usually result in efficient crosslinking. SASD (Product # 27716) is a unique sulfo-NHS-ester, photoactivatable phenyl azide that is both iodinatable and cleavable that allows for detection and analysis of small quantities of protein. For more information on this type of application for crosslinkers, refer to the free Protein:Protein Interaction Technical Handbook (Product # 1601190).

Creation of immunotoxins

Specific antibodies can be covalently linked to toxic molecules and then used to target antigens on cells. Often these antibodies are specific for tumor-associated antigens. Immunotoxins are brought into the cell by surface antigens and, once internalized, they proceed to kill the cell by ribosome inactivation or other means. The type of crosslinker used to make an immunotoxin can affect its ability to locate and kill the appropriate cells. For immunotoxins to be effective, the conjugate must be stable *in vivo*. In addition, once the immunotoxin reaches its target, the antibody must be separable from the toxin to allow the toxin to kill the cell. Thiol-cleavable, disulfide-containing conjugates have been shown to be more cytotoxic to tumor cells than noncleavable conjugates of ricin A immunotoxins. Cells are able to break the disulfide bond in the crosslinker, releasing the toxin within the targeted cell.

SPDP (Product # 21857) is a reversible NHS-ester, pyridyl disulfide crosslinker used to conjugate amine-containing molecules to sulfhydryls. For several years, this has been the "workhorse" crosslinker for production of immunotoxins. The amine-reactive NHS-ester is usually reacted with the antibody first. In general, toxins do not contain surface sulfhydryls; therefore, sulfhydryls must be introduced into them by reduction of disulfides, which is common for procedures involving ricin A chain and abrin A chain, or through chemical modification reagents. A second SPDP molecule can be used for this purpose and is reacted with amines on the immunotoxin, then reduced to yield sulfhydryls. Another chemical modification reagent that is commonly used for production of immunotoxins is 2-iminothiolane, also known as Traut's Reagent (Product # 26101). Traut's Reagent reacts with amines and yields a sulfhydryl when its ring structure opens during the reaction.

Carrier protein conjugation, the creation of immunogens

Many crosslinkers are used for making conjugates for use as immunogens (Table 2). The best crosslinker to use depends on the functional groups present on the hapten and the ability of the hapten-carrier conjugate to function successfully as an immunogen after its injection. Carbodiimides are good choices for producing peptide-carrier protein conjugates because both proteins and peptides usually contain several carboxyls and primary amines. Carbodiimides such as EDC react with carboxyls first to yield highly reactive unstable intermediates that can then couple to primary amines. Often peptides are synthesized with terminal cysteines to enable attachment to supports or to carrier proteins using sulfhydryl-/amine-reactive, heterobifunctional crosslinkers. This method can be very efficient and yield an immunogen that is capable of eliciting a good response upon injection. For more information on preparation of immunogen conjugates, refer to the free Antibody Production and Purification Technical Handbook (Product # 1601092).

Suggested Reading

For more information concerning accurate measurements of 32 popular crosslinkers using stochastic dynamics calculations, see the following reference:

Houk, K.N., *et al.* (2001). Quantitative evaluation of the length of homobifunctional protein crosslinking reagents used as molecular rulers. *Protein Sci.* **10**, 1293-1304.

Table 2. Crosslinkers commonly used to produce immunogens.

Crosslinker	Reactivity	Product #
EDC	Amine/Carboxyl	22980, 22981
SMCC	Amine/Sulfhydryl	22360
Sulfo-SMCC	Amine/Sulfhydryl	22322
MBS	Amine/Sulfhydryl	22311
Sulfo-MBS	Amine/Sulfhydryl	22312
SMPB	Amine/Sulfhydryl	22416
Sulfo-SMPB	Amine/Sulfhydryl	22317
GMBS	Amine/Sulfhydryl	22309
Sulfo-GMBS	Amine/Sulfhydryl	22324

Solid-phase immobilization

Proteins, peptides and other molecules can be immobilized onto solid supports for affinity purification of proteins or for sample analysis. The supports may be nitrocellulose or other membrane materials, polystyrene plates or balls, agarose, beaded polymers, or glass slides. Some supports can be activated for direct coupling to a ligand. Other supports are made with nucleophiles or other functional groups that can be linked to proteins using crosslinkers. Carbodiimides such as DCC (Product # 20320) and EDC (Product # 22980, 22981) are very useful for coupling proteins to carboxy- and amine-activated glass, plastic and agarose supports. Carbodiimide procedures are usually one-step methods; however, two-step methods are possible if reactions are performed in organic solvents, or if NHS (Product # 24500) or Sulfo-NHS (Product # 24510) is used to enhance the reaction. EDC is useful for coupling ligands to solid supports and to attach leashes onto affinity supports for subsequent coupling of ligands. Useful spacers are diaminodipropylamine (DADPA), ethylenediamine, hexanediamine, 6-amino-caproic acid and any of several amino acids or peptides. Spacer arms help to overcome steric effects when the ligand is immobilized too near the matrix to allow access by the receptor. Steric effects are usually most pronounced when the ligand is a small molecule.

Heterobifunctional crosslinkers that can be reacted in two-steps are often more useful and efficient for producing solid-phase supports than homobifunctional crosslinkers. Amine-activated supports can be converted to sulfhydryl-reactive supports using NHS-ester maleimide crosslinkers such as Sulfo-SMCC (Product # 22322). For some compounds that are difficult to immobilize, it may be possible to use NHS-ester, photoactivatable, phenyl azides to attach them to amine-activated supports. The photoactivatable phenyl azide becomes reactive once it is exposed to the appropriate wavelength and is able to nonselectively couple to almost any ligand.

The crosslinkers DMP (Product # 20666) and DSS (Product # 21555) are used to immobilize antibodies on Protein A or Protein G supports for antigen purification. After the antibody binds to the Fc-binding proteins, the antibody is oriented so that the Fab region is available for antigen binding. DSS or DMP is applied to the bound antibody column to link the two proteins through primary amines. The Seize® X Immunoprecipitation Kits (e.g., Product # 45215) are based on this chemistry. For more information on solid-phase immobilization, refer to the free Affinity Purification Technical Handbook (Product # 1600976).

Protein:protein conjugates

One of the most-used applications for crosslinkers is the production of protein:protein conjugates. Conjugates are often prepared by attachment of an enzyme, fluorophore or other molecule to a protein that has affinity for one of the components in the biological system being studied. Antibody-enzyme conjugates (primary or secondary antibodies) are among the most common protein:protein conjugates used. Although secondary antibody conjugates are available and relatively inexpensive, enzyme-labeled primary antibodies are usually expensive and can be difficult to obtain.

Many reagents are used for the production of antibody-enzyme conjugates. Glutaraldehyde conjugates are easy to make, but they often yield conjugates that produce high background in immunoassays. Carbohydrate moieties can be oxidized and then coupled to primary amines on enzymes in a procedure called reductive alkylation or amination. These conjugates often result in less background in enzyme immunoassays and are relatively easy to prepare; however, some self-conjugation of the antibody may occur. Homobifunctional NHS-ester or imidoester crosslinkers may be used in a one-step protocol but polymerization and self-conjugation are also likely. Homobifunctional sulfhydryl-reactive crosslinkers such as BMH (Product # 22330) and DPDPB (Product # 21702) may be useful if both proteins to be conjugated contain sulfhydryls.

Heterobifunctional crosslinkers are perhaps the best choices for antibody-enzyme or other protein:protein crosslinking. Unwanted self-conjugation inherent when using homobifunctional NHS-ester reagents or glutaraldehyde can be avoided by using a reagent such as SMCC (Product # 22360) or Sulfo-SMCC (Product # 22322). Sulfo-SMCC is first conjugated to one protein, and the second is thiolated with SATA (Product # 26102) or Traut's Reagent (Product # 26101), followed by conjugation. Alternatively, disulfides in the protein may be reduced, and the two activated proteins are incubated together to form conjugates free of dimers of either protein. Any of the other NHS-ester, maleimide or pyridyl disulfide crosslinkers can be substituted for Sulfo-SMCC in this reaction scheme. Heterobifunctional photoactivatable phenyl azide crosslinkers are seldom used for making protein:protein conjugates because of low conjugation efficiencies.

Another strategy for creating specific protein conjugates without the risk of self-conjugation takes advantage of a two-step NHS-ester/hydrazide and NHS-ester/aldehyde (e.g., Product # 22411, 22419) chemistry. In this strategy, one component of the conjugate is activated with the NHS-ester/hydrazide (e.g., Product # 22411), while in a separate reaction, the other component is activated with the NHS-ester/aldehyde (e.g., Product # 22419). The hydrazide- and aldehyde-activated components are then mixed together and spontanteously react to form the specific conjugate. These same reagents are also useful for activating surfaces to which a biomolecule is to be bound.

DNA/RNA crosslinking to proteins

Crosslinking of DNA or RNA to proteins is more limited because the reactivities of most crosslinkers favor protein:protein crosslinking over protein:DNA crosslinking. To assist in these crosslinking methods, DNA probes are often synthesized with primary amines or thiols attached to specific bases. After insertion of the bases into DNA, amine- or sulfhydryl-reactive crosslinkers can be used for their conjugation to proteins. EDC has been reportedly used to crosslink RNA to ribosomal protein subunits. Other specialized chemistries are reviewed in Hermanson's book, *Bioconjugate Techniques* (Product # 20002).

Label transfer

Label transfer involves crosslinking interacting molecules (i.e., bait and prey proteins) with a labeled crosslinking agent and then cleaving the linkage between bait and prey such that the label remains attached to the prey (Figure 1, page 11). This method allows a label to be transferred from a known protein to an unknown, interacting protein. The label can then be used to purify and/or detect the interacting protein. Label transfer is particularly valuable because of its ability to identify proteins that interact weakly or transiently with the protein of interest. New non-isotopic reagents and methods continue to make this technique more accessible and simple to perform by any researcher.

Traditional label transfer reagents

The earliest examples of label transfer reagents incorporated a photoreactive phenyl azide group that contained a hydroxy-phenyl modification on the ring. The phenolic hydroxyl activates the ring for substitution reactions to occur ortho or para to its position. These compounds can be radioiodinated using typical oxidation reagents such as chloramine T or IODO-BEADS® Iodination Reagent (see the Protein Structure section of the Pierce catalog for more information on iodination). Iodination of the crosslinker with ^{125}I prior to its use will result in a radioactive label transfer reagent that can tag an unknown interacting protein with a radiolabel after cleavage of the crosslinker's spacer arm.

In practice, the crosslinker is first radioiodinated and then reacted with a bait protein, typically through available amine groups. This modified protein is then introduced into a sample and allowed to interact with other proteins. The sample is exposed to UV light to photo-crosslink the interacting complex. At this point, the label can facilitate detection of the interacting proteins or the complex can be cleaved and the radiolabel transferred to the protein interacting with the bait. The now radiolabelled, unknown protein(s) can be detected by autoradiography after separation by electrophoresis and Western transfer.

The first reagents employed using this method were bifunctional. They were designed such that the photoreactive moiety bears the transferable label. These molecules are either amine-reactive or sulfhydryl-reactive and are labeled radioisotopically with ^{125}I. More recent offerings have been prepared as trifunctional reagents that more adequately segregate the reactive sites from the label. These trifunctional reagents can be designed to include non-radioisotopic labels such as biotin.

SASD and APDP: radiolabel transfer reagents

SASD and APDP (Product # 27716 and 27720, respectively) are heterobifunctional crosslinkers containing a photoreactive group that can be labeled with ^{125}I. They differ only in the functional group against which they are directed on the bait protein. SASD contains the amine-reactive sulfo-NHS group. APDP contains the sulfhydryl-reactive pyridyl-dithio group. The sulfhydryl-reactive group of APDP offers the advantage of allowing the course of the bait protein coupling to be monitored by following the loss of the 2-pyridyl-thione moiety (leaving group). The 2-pyridyl-thione can be detected at 343 nm (extinction coefficient: $8.08 \times 10^3\ M^{-1}cm^{-1}$).

Disadvantages of traditional bifunctional label transfer reagents

Although these reagents have been used successfully to obtain data on protein interactions, they possess some inherent deficiencies compared to trifunctional reagents designed for label transfer applications. The user should be aware of the following characteristics of these reagents.

1. Photoreactive and labeled chemical groups are the same.
2. They require labeling with ^{125}I before use, and the efficiency of label incorporation is low.
3. The photoactivation step can result in several unproductive pathways that lower crosslinking yield between bait and prey.
4. The ^{125}I label can be released during the light reaction, causing nonspecific labeling of the protein(s) in the mix.

SAED: fluorescent label transfer reagent

Subsequent designs of bifunctional label transfer reagents used nonradioactive labels to avoid the safety issues posed by ^{125}I. Fluorescent constituents designed into cleavable photoreactive crosslinkers make possible transfer of a fluorescent label to an unknown interacting protein. An example of this type of reagent that incorporates a coumarin group is SAED (Product # 33030), which has been substituted with an azido group on the aromatic, photoreactive ring. The reagent is non-fluorescent prior to exposure to UV light, but upon photolyzing and coupling to interacting proteins, it becomes highly fluorescent. The reagent also has a disulfide bond that can be reduced, resulting in cleavage of the crosslinked proteins and transfer of the label to the unknown interacting species. In this case, the fluorescently labeled interacting proteins can be followed in cells to determine the site of interactions or the fate of the proteins after interacting.

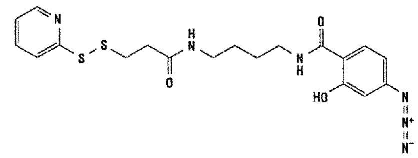
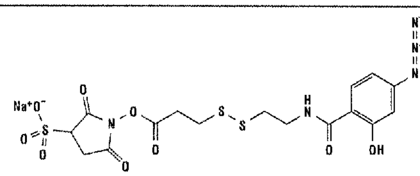
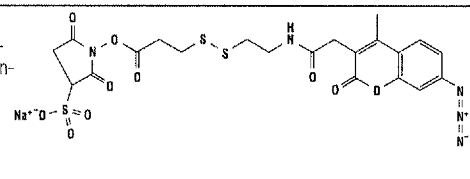
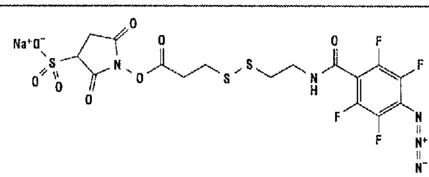
SFAD: fluorine label transfer reagent

SFAD (Product # 22719) is a heterobifunctional crosslinker containing an amine-reactive NHS-ester moiety at one end and a photoreactive perfluorophenyl azide moiety at the other end, separated by a cleavable disulfide bond. The presence of fluorine allows the label transfer process to be monitored by ^{19}F NMR. Also the improved stability of the perfluoroaryl nitrene-reactive intermediate provides additional efficiency of insertion into C-H bonds compared to the amine nucleophile reaction typical of non-fluorinated aryl nitrenes that quickly undergo ring expansion.

Label transfer reagents

Bifunctional label transfer reagents

Heterobifunctional, photoreactive, thiol-cleavable label transfer reagents enable the tagging of a prey protein. The photolysis wavelengths for these reagents are in the range between 320-400 nm, limiting damage to biomolecules by irradiation.

Product #	Product Name	Structure	Key Features	Ref.	Pkg. Size
27720	APDP *N*-[4-(*p*-Azido-salicylamido) butyl]-3′-(2′-pyridyldithio) propionamide **M.W.** 446.55 **Spacer Arm** 21.0 Å		• Radioiodinatable between $-N_3$ and –OH group of phenyl ring • –SH-reactive • Reaction monitored at 343 nm • Membrane permeable	1-5	50 mg
27716	SASD Sulfosuccinimidyl-2-[*p*-azidosalicylamido] ethyl-1,3′-dithiopropionate **M.W.** 541.51 **Spacer Arm** 18.9 Å		• Radioiodinatable between $-N_3$ and –OH group of phenyl ring • $-NH_2$-reactive • Water-soluble	3,6-13	50 mg
33030	SAED Sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamido)ethyl-1,3′dithiopropionate **M.W.** 621.6 **Spacer Arm** 23.6 Å		• Water-soluble • Amine-reactive • Photoreactive • Prey protein tracked by fluorescence • Ex: 345-350 nm, Em: 440-460 nm • No radiolabeling required • AMCA moiety exhibits large Stokes shift	16,17	5 mg
27719	SFAD Sulfosuccinimidyl(perfluoroazidobenzamido)ethyl-1,3′-dithiopropionate **M.W.** 597.48 **Spacer Arm** 14.6 Å		• Improved photoconjugation efficiency • Photolyzes at 320 nm • Label transfer monitored by ^{19}F NMR • Water-soluble • Cleavable • Amine-reactive	9,10,14,15	50 mg

Compatible products for addition of ^{125}I to APDP or SASD.

Related Pierce Products:

Product #	Description	Pkg. Size
28601	IODO-GEN® Pre-Coated Iodination Tubes (12 mm x 75 mm glass test tubes coated with 50 µg IODO-GEN® Iodination Reagent in 100 µl volume)	10 tubes/pkg.
28665	IODO-BEADS® Iodination Reagent (*N*-Chloro-benzenesulfonamide modified non-porous polystyrene beads)	50/pkg.
28666	IODO-BEADS® Iodination Reagent	250/pkg.
28600	IODO-GEN® Iodination Reagent (1,3,4,6-Tetrachloro-3α,6α-diphenylglycoluril)	1 g

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1. React available amine groups on a purified Bait Protein **(Protein 1)** with Sulfo-NHS containing Biotin Label Transfer Reagent [pH 7-9, in the dark, 30 minutes at RT].
2. Introduce Biotinylated Bait Protein to Prey Protein **(Protein 2)** containing sample under conditions which promote favorable binding. Incubate in the dark 30-60 minutes.
3. Capture the Bait:Prey complex by irradiating the photoreactive aryl azide group with UV light.
4. Reduce the disulfide bond using DTT. Transfer the Biotin label from the **Bait Protein 1** to the **Bait Protein 2**.
5. Reduced sample is applied to a gel and separated by electrophoresis. Transfer proteins to a membrane. Detect Bait or Prey Proteins with the appropriate antibodies or Streptavidin-HRP.

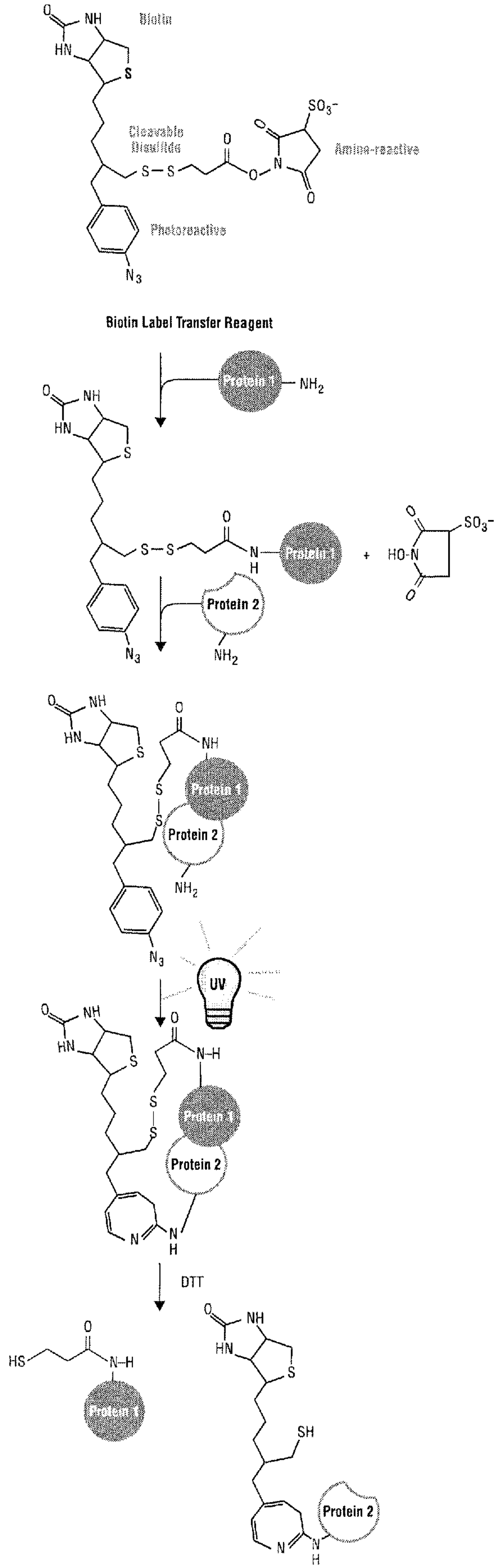

Figure 1. General scheme for label transfer reactions.

1

Sulfo-SBED: ProFound™ Label Transfer Protein:Protein Interaction Reagent

Label transfer reagents can also have biotin built into their structure. This type of design allows the transfer of a biotin tag to an interacting protein after cleavage of a cross-bridge. Sulfo-SBED* (Product # 33033) is an example of such a trifunctional reagent (Figure 2). It contains an amine-reactive sulfo-NHS-ester on one arm (built off the α-carboxylate of the lysine core), a photoreactive phenyl azide group on the other side (synthesized from the α-amine) and a biotin handle (connected to the ε-amino group of lysine). The arm containing the sulfo-NHS-ester has a cleavable disulfide bond, which permits transfer of the biotin component to any captured proteins.

In use, a bait protein first is derivatized with Sulfo-SBED through its amine groups, and the modified protein is allowed to interact with a sample. Exposure to UV light (300-366 nm) couples the photoreactive end to the nearest available C-H or N-H bond in the bait:prey complex, resulting in covalent crosslinks between bait and prey. Upon reduction and cleavage of the disulfide spacer arm, the biotin handle remains attached to the protein(s) that interacted with the bait protein, facilitating isolation or identification of the unknown species using streptavidin, NeutrAvidin™ Protein or monomeric avidin.

The architecture of this trifunctional label transfer reagent differs substantially from the bifunctional counterparts discussed above. The advantages become almost immediately apparent just by examining the structure.

The reactive moieties are well-segregated within Sulfo-SBED. Most importantly, with a biotin label designed into Sulfo-SBED, radiolabeling with ^{125}I is no longer necessary. The biotin label can be used to significant advantage in a label transfer application. For example, biotin can operate as a handle for purification of the prey protein or prey protein fragments or as a detection target using streptavidin-HRP and colorimetric or chemiluminescent substrates.

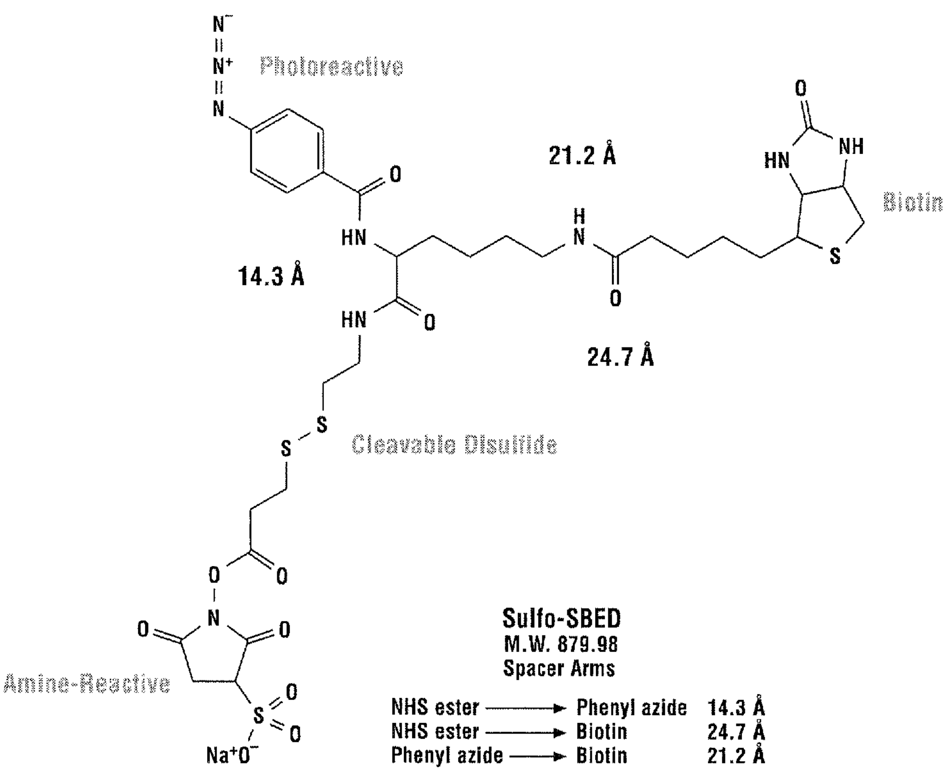

Figure 2. Structure of Sulfo-SBED.

**Sulfo-SBED Technology is protected by U.S. patent # 5,532,379.*

Applications for Sulfo-SBED

Since the first availability of this patented reagent in 1994, the number of literature references for use of Sulfo-SBED in protein interaction-related applications has grown rapidly. Published applications show how Sulfo-SBED can used to:

- Define interactions of complexes with activator domains[1]
- Clarify the mechanism of protein complex assembly[2]
- Convert to a sulfhydryl-reactive trifunctional reagent to map interactions[3]
- Study docking site and factor requirements for binding[4]
- Describe binding contacts of interactors[5]
- Confirm recognition of a specific phosphoepitope[6]
- Search for putative binding partners[7]
- Gain insight into chaperone-mediated refolding interactions[8]
- Investigate mechanism of protein interaction[9]
- Facilitate receptor activity-directed affinity tagging (re-tagging)[10]
- Detect low-abundance protein receptors[11]
- Find protein:carbohydrate interactions[11]
- Understand drug-receptor interactions[12]
- Quantitate triple helix-forming oligonucleotides[13]

Routes to determining the prey protein identification using Sulfo-SBED are outlined schematically in Figure 3. Note that the biotin label is a purification handle for captured prey protein. In the trypsin digestion strategy, the peptide(s) trapped can offer information relating to the binding interaction interface. The biotin-labeled prey protein or prey protein peptides recovered as result of the strategies outlined below can be subjected to several detection and identification options designed to discover the identity of the prey protein.

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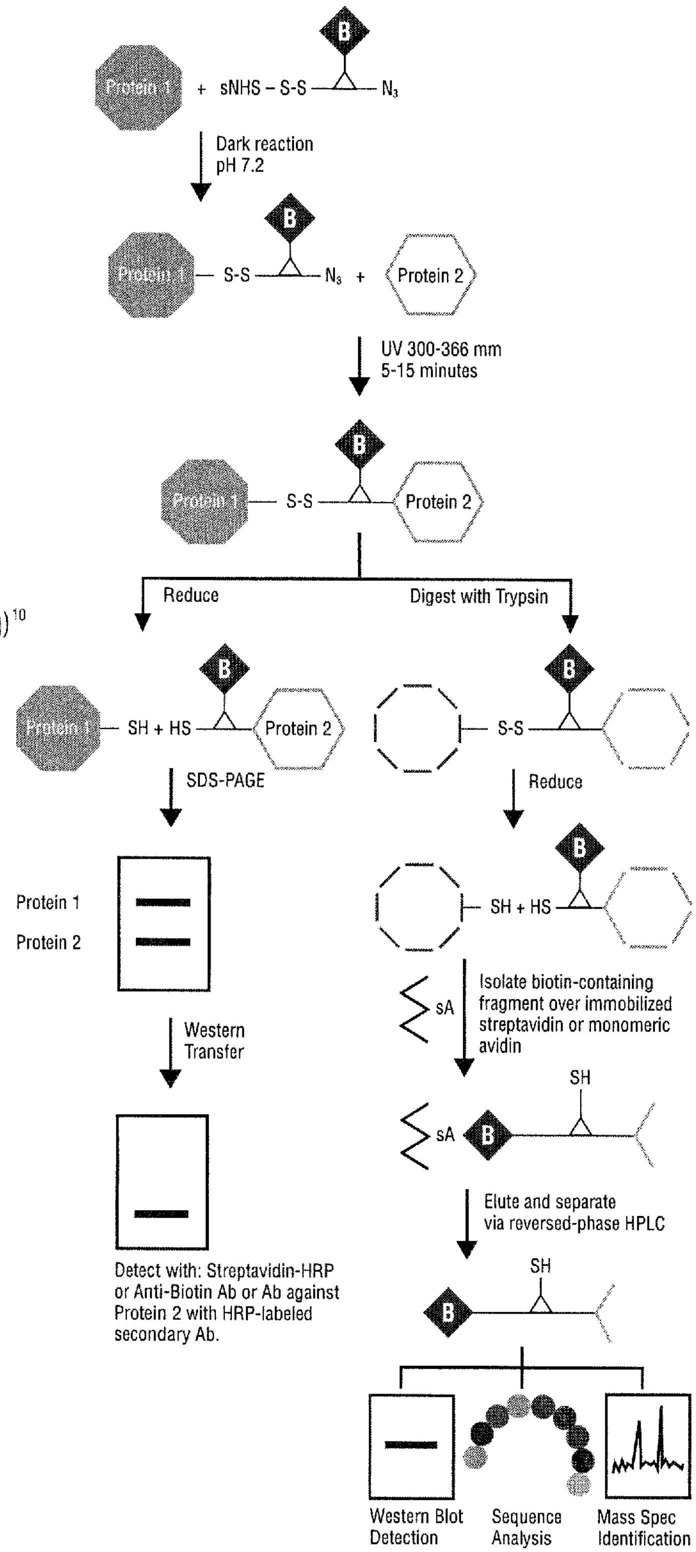


Legend: **sNHS** Sulfo *N*-Hydroxy succinmide ester; **S-S** Disulfide bond; **N_3** Phenyl azide; **B** Biotin

Figure 3. Applications of Sulfo-SBED in protein interaction studies.

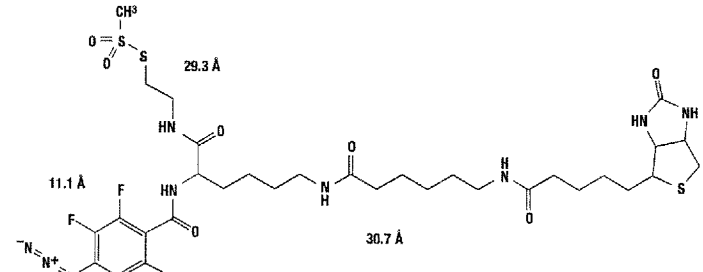
Crosslinkers are available in bulk quantities for manufacturing applications

Tel: 800-874-3723 or 815-968-0747 www.piercenet.com/xlink95d

Mts-Atf-Biotin Label Transfer Reagents

Sulfhydryl-directed, photoreactive biotin label transfer reagents.

These two new biotin-containing reagents incorporate the benefits of the sulfhydryl-specific methanethiosulfonate (Mts) group and the high-yielding photoreactive tetrafluorophenyl-azide moiety. By combining these reactive groups with a biotin tag, powerful new reagents for protein interaction analysis were created. Purified bait protein is labeled at reduced cysteine residues, then allowed to form an interaction complex with the prey protein. When exposed to UV-light, the photoreactive group activates to form covalent bonds to adjacent sites on the prey protein. Reducing the disulfide-bond releases the bait protein and leaves the biotin label on the prey.

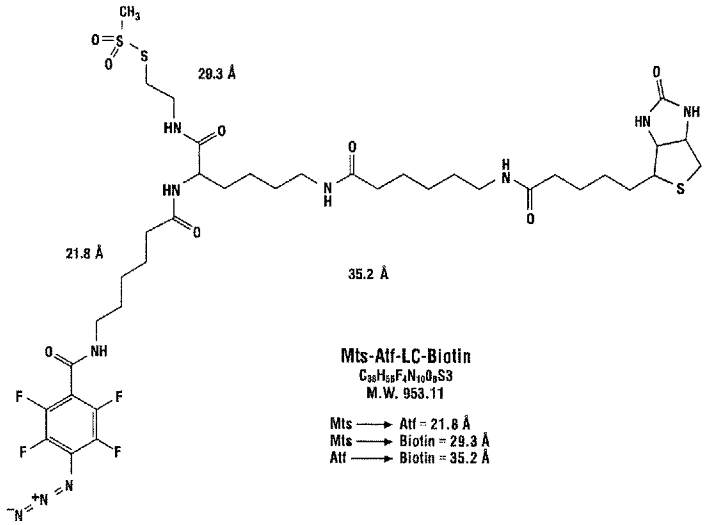


Mts-Atf-Biotin
$C_{32}H_{45}F_4N_9O_7S_3$
M.W. 839.95

Mts ⟶ Atf = 11.1 Å
Mts ⟶ Biotin = 29.3 Å
Atf ⟶ Biotin = 30.7 Å

Highlights:

- Mts moiety is highly specific for the sulfhydryl (–SH) group that occurs in the side chain of reduced cysteine residues, enabling precise, rapid and quantitative labeling of the bait protein
- Tetrafluorophenyl azide moiety reacts three- to four-times more efficiently than regular phenyl azide moieties, increasing the likelihood of capturing sufficient bait:prey complex to detect
- Sulfinic acid byproducts of the Mts reaction do not interfere with disulfide bond formation or the activity of the bait protein and decomposes quickly to a volatile low molecular weight product
- Disulfide bond spacer arm connecting bait and prey proteins is easily reversed with commonly used reducing agents DTT, 2-mercaptoethanol or TCEP
- Mts reaction and photoreaction are compatible with physiologic buffer conditions required for most protein interactions
- Long chain (LC) and short chain versions are offered, allowing one to more precisely explore interaction distances

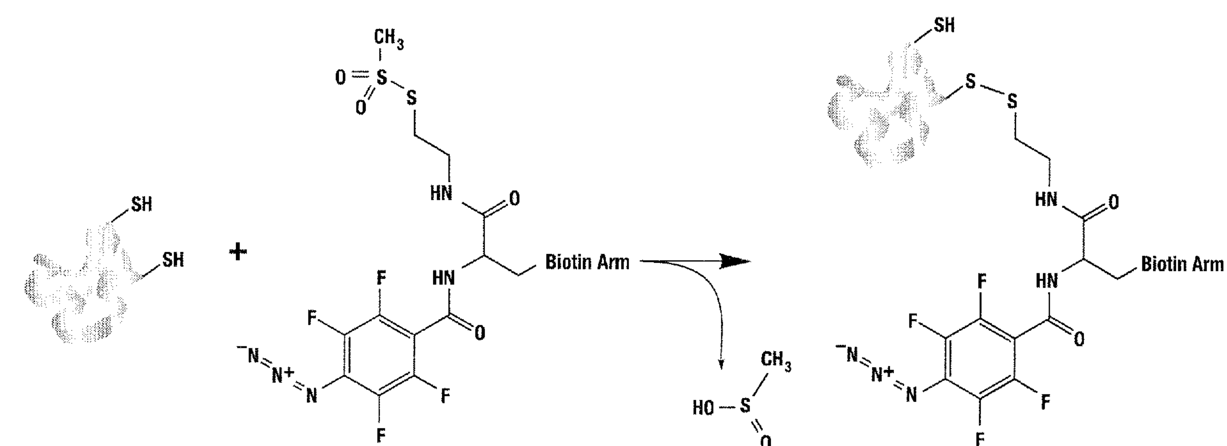


Mts-Atf-LC-Biotin
$C_{38}H_{56}F_4N_{10}O_8S_3$
M.W. 953.11

Mts ⟶ Atf = 21.8 Å
Mts ⟶ Biotin = 29.3 Å
Atf ⟶ Biotin = 35.2 Å

Ordering Information

Product #	Description	Pkg. Size
33093	Mts-Atf-Biotin Label Transfer Reagent 2-[*N*2-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-*N*6-(6-biotinamidocaproyl)-L-lysinyl]ethylmethanethiosulfonate	5 mg
33083	Mts-Atf-LC-Biotin Label Transfer Reagent 2-{*N*2-[*N*6-(4-Azido-2,3,5,6-tetrafluorobenzoyl-6-aminocaproyl)-*N*6-(6-biotinamidocaproyl)-L-lysinylamido)]ethylmethanethiosulfonate	5 mg



Bait protein containing sulfhydryls | **Mts-Atf-Biotin** | **Methylsulfinic acid** | **Activated Bait Protein**

Figure 4. Reaction of Mts-Atf-Biotin with bait protein containing sulfhydryls (reduced disulfide bonds). Once desalted to remove excess nonreacted Mts-Atf-Biotin and byproducts (methylsulfinic acid), the activated bait protein may be allowed to interact with other proteins (the prey) and then crosslinked together by UV-activiation of the tetrafluorophenyl azide group. If desired, the disulfide bond in the Mts-Atf-Biotin may be cleaved with a reducing agent, transferring the biotin label to the prey protein.

Structure determination with heavy/light crosslinker pairs

Recently, chemical crosslinking, combined with high-resolution mass spectrometry, has emerged as a strategy to obtain low-resolution three-dimensional structural data of protein structures and protein interfaces in complexes from low quantities of proteins within a relatively short time. However, identification of the large number of crosslinking sites from the complex mixtures generated by chemical crosslinking remains a challenging task.

By incorporating an isotopic label into the crosslinking reagent, thus conducting linking and labeling in one step, the crosslinked peptides are identified easily in the presence of the numerous unmodified tryptic peptides. The strategy requires the availability of both "light" or hydrogen-containing and "heavy" or discretely substituted deuterium analogs of crosslinking agents. Heavy and light analogs are reacted simultaneously with the target protein or protein complex. Use of heavy and light crosslinkers in this application dramatically simplifies identification of the peptides resulting from the coupling reactions. Application of a 1:1 ratio of two identical crosslinking agents differing only in the number of deuterium atoms in their chemical composition (e.g., d_4 vs d_0) facilitates identification of low-abundance crosslinked peptides. Isotopic MS patterns, differing by four mass units after enzymatic digestion of the crosslinked protein or protein complex, identifies the crosslinked products.

Further analysis of the reaction products resulting from the simultaneous reaction of these heavy and light crosslinkers with a target protein or protein complex is accomplished by MALDI-TOF-MS, ESI-LC/MS/MS or ESI-FTICR-MS. The results positively identify the crosslinked peptides. Distance constraints provided by these data can yield low-resolution three-dimensional structure information that can be used to create structural models of the protein in solution. Intermolecular crosslinking of an interacting protein complex and subsequent MS analysis have been successfully applied to determine the contact surfaces of binding partners in a protein complex.[2-6]

Heavy/light crosslinker pairs

BS^2G and BS^3 are water-soluble, homobifunctional sulfonated *N*-hydroxysuccinimide esters (Sulfo-NHS esters) with a 7.7 Å and 11.4 Å spacer arm that can act as molecular rulers for estimation of spatial relationships in protein structure-function studies. The reagents described here are the deuterated and non-deuterated analogs of BS^2G and BS^3. These reagents react efficiently with primary amine groups ($-NH_2$) at pH 7-9 to form stable amide bonds. Proteins generally contain several primary amines in the form of lysine residue side chains and the N-terminus of each polypeptide that are available as targets for the NHS ester-reactive group. BS^2G-d_4 and BS^3-d_4 react identically to their H-substituted counterparts. These reagents are supplied as a sodium salt and are soluble in water at a concentration up to 10 mM.

1

Ordering Information

Product #	Description	Pkg. Size
21590	BS^3-d_0 *Bis*(sulfosuccinimidyl)suberate-d_0	10 mg
21595	BS^3-d_4 *Bis*(sulfosuccinimidyl)2,2,7,7-suberate-d_4	10 mg
21610	BS^2G-d_0 *Bis*(sulfosuccinimidyl)glutarate-d_0	10 mg
21615	BS^2G-d_4 *Bis*(sulfosuccinimidyl)2,2,4,4-glutarate-d_4	10 mg

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Kalkhof, S., *et al.* (2005). Chemical crosslinking and high-performance Fourier transform ion cyclotron resonance mass spectrometry for protein interaction analysis: application to a calmodulin/target peptide complex. *Anal. Chem.* **77**, 495-503.

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Single-step vs. multi-step reactions

Homo- and heterobifunctional crosslinkers

Crosslinkers can be either homobifunctional or heterobifunctional. Homobifunctional crosslinkers have two identical reactive groups and often are used in one-step reaction procedures to crosslink proteins, to each other or to stabilize quaternary structure, in solution. Even when conjugation of two different proteins is the goal, one-step crosslinking with homobifunctional reagents often results in self-conjugation, intramolecular crosslinking and/or polymerization.

Heterobifunctional crosslinkers possess two different reactive groups that allow for sequential (two-stage) conjugations, helping to minimize undesirable polymerization or self-conjugation. Heterobifunctional reagents can be used when modification of amines is problematic. Amines are sometimes present at the active sites of proteins and modification of these may lead to activity loss. Other moieties such as sulfhydryls, carboxyls, phenols and carbohydrates may be more appropriate targets. A two-step strategy allows a protein that can tolerate the modification of its amines to be coupled to a protein or other molecule having different accessible groups. Crosslinkers that are amine-reactive at one end and sulfhydryl-reactive at the other end are especially useful in this regard.

In sequential procedures, heterobifunctional reagents are reacted with one protein using the most labile group of the crosslinker first. After removing excess nonreacted crosslinker, the modified first protein is added to a solution containing the second protein where reaction through the second reactive group of the crosslinker occurs. The most widely used heterobifunctional crosslinkers are those having an amine-reactive succinimidyl ester (e.g., NHS-ester) at one end and a sulfhydryl-reactive group on the other end. The sulfhydryl-reactive groups are usually maleimides, pyridyl disulfides and α-haloacetyls. The NHS-ester reactivity is less stable in aqueous solution and is usually reacted first in sequential crosslinking procedures. NHS-esters react with amines to form amide bonds. Carbodiimides are zero-length crosslinkers (e.g., EDC, Product # 22980, 22981) and effect direct coupling between carboxylates (–COOH) and primary amines ($-NH_2$) and have been used in peptide synthesis, hapten-carrier protein conjugation, subunit studies and protein:protein conjugation.

Other heterobifunctional reagents have one reactive group that is photoreactive rather than thermoreactive. These have distinct advantages in protein:protein interaction studies and in cases in which the availability of thermoreactive targetable functional groups is unknown. This reactivity allows for specific attachment of the labile thermoreactive group first; subsequently, conjugation to any adjacent N-H or C-H sites may be initiated through the photoreactive group by activation with UV light.

The reactivity of the photochemical reagent allows for formation of a conjugate that may not be possible with a group-specific reagent. The efficiency of photoreactive crosslinkers is low, and yields of 10% are considered acceptable. However, SFAD (Product # 27719) is a photoactivatable reagent that contains a perfluorophenyl azide with an insertion efficiency of 70%.

Bioconjugate Toolkit

The next generation of biomolecule immobilization/conjugation.

The modification of biomolecules or surfaces with hydrazine/hydrazide- and aldehyde-reactive moieties provides a small molecule conjugation chemistry that is easy to use and applicable to almost any conjugation scheme. Simple mixing of a hydrazine-/hydrazide-modified biomolecule with an aldehyde-modified biomolecule yields the desired conjugate. The leaving group in the reaction is water and no reducing agents (e.g., sodium cyanoborohydride) are required to stabilize the bond. Unlike other small molecule conjugation methods such as maleimido/thiol chemistry, molecules or surfaces modified with either hydrazine/hydrazide or aldehyde moieties have extended stabilities in aqueous environments. These groups can be incorporated on any surface and remain active without special handling requirements.

Highlights:

- No hazardous reducing agents (e.g., sodium cyanoborohydride) required
- Long-term stability
  - Biomolecules and surfaces modified with hydrazine/hydrazide or aldehyde groups can be prepared, stored and used when needed
  - Hydrazine (SANH)-reactive moieties are stable for several months
  - Hydrazide (SHTH)-reactive moieties are stable indefinitely
- Can be applied to almost any conjugation scheme
- Easy to use
  - Simple mixing of a hydrazine-/hydrazide-modified biomolecule with an aldehyde-modified biomolecule yields the desired conjugate
  - Reaction is performed in aqueous buffered solutions (pH 4.5-7.4) or organic solvents with high efficiency
  - Hydrazide (SHTH)-reactive moieties are stable indefinitely
- Highly efficient coupling chemistry
- Reactive moieties do not lead to nonspecific interactions
- Conjugation does not result in inter-subunit crosslinking

Ordering Information

Product #	Description	Pkg. Size
22400	SANH (Succinimidyl 4-hydrazinonicotinate acetone hydrazone) Application: used to convert primary amines to hydrazinopyridine moieties	25 mg
22405	C6-SANH (C6-succinimidyl 4-hydrazinonicotinate acetone hydrazone) Application: SANH with extended six carbon linker	25 mg
22411	SHTH (Succinimidyl 4-hydrazidoterephthalate hydrocholoride) Application: used to convert primary amines to aromatic hydrazide moieties	25 mg
22419	SFB (Succinimidyl 4-formylbenzoate) Application: used to convert primary amines to benzaldehyde moieties	100 mg
22423	C6-SFB (C6-Succinimidyl 4-formylbenzoate) Application: SFB with extended six carbon linker	25 mg

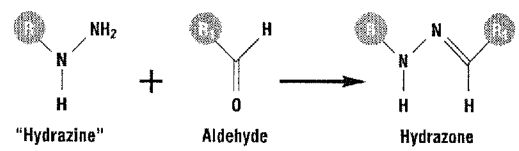

Figure 5. The hydrazine/carbonyl reaction.

To view structures of these products, see Appendix II.

Controlled Protein-Protein Cross-Linking Kit

Contains everything you need to crosslink two proteins and do it successfully.

Highlights:

- Reliable noncleavable heterobifunctional crosslinking agent offers proven chemistry, highly stable intermediates and efficient formation of the target conjugate
- Includes disulfide (S-S) reductants and thiolation reagents
- Ellman's Reagent provides the option to monitor reaction sufficiently

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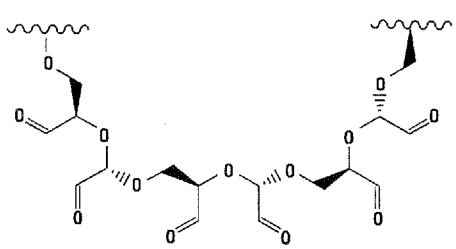
Ordering Information

Product #	Description	Pkg. Size
23456	Controlled Protein-Protein Cross-Linking Kit	Kit
	Includes: Sulfo-SMCC Crosslinking Agent	2 mg
	10X Activation/Conjugation Buffer	20 ml
	BupH™ Phosphate Buffered Saline	2 packs
	2-Mercaptoethylamine•HCl	6 mg
	Immobilized Reductant	0.2 ml
	SATA	2 mg
	Dimethylformamide (DMF)	1 ml
	Hydroxylamine•HCl	5 mg
	D-Salt™ Dextran Desalting Column	2 x 10 ml
	Column Extender	2 ea.
	Cysteine•HCl	20 mg
	Ellman's Reagent	2 mg

Activated Dextran Coupling Kit

Couple amine-containing ligands and biomolecules.



Ordering Information

Product #	Description	Pkg. Size
20890	Aldehyde-Activated Dextran Coupling Kit	Kit
	Includes: Aldehyde-Activated Dextran*	5 x 5 mg
	[CHO Loading: ~300 moles mole of dextran]	
	Sodium Cyanoborohydride	190 mg
	BupH™ Phosphate Buffered Saline	1 pack

**The average molecular weight of dextran used in these preparations is 40 kDa.*

Reference

Mallia, A.K. and Vigna, R.A. (1998). *Previews* **1(4)**, 16-17.

Amine-reactive chemistries

Imidoesters

Imidoester crosslinkers react with primary amines to form amidine bonds. The resulting amidine is protonated and, therefore, has a positive charge at physiological pH (Figure 6). Imidoester homobifunctional crosslinkers have been used to study protein structure and molecular associations in membranes and to immobilize proteins onto solid-phase supports. They also have been examined as a substitute for glutaraldehyde for tissue fixation. Imidoesters can penetrate cell membrane and crosslink proteins within the membrane to study membrane composition, structure and protein:protein and protein:lipid interactions. These crosslinkers have also been used to determine or confirm the number and location of subunits within multi-subunit proteins. In these experiments, large molar excesses of crosslinker (100- to 1,000-fold) and low concentrations of protein (1 mg/ml) are used to favor intramolecular over intermolecular crosslinking.

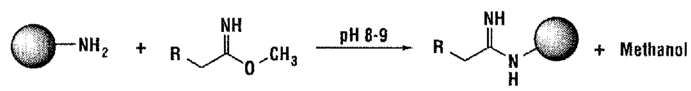


Figure 6. Imidoester reaction scheme.

Imidoester crosslinkers react rapidly with amines at alkaline pH, but have short half-lives. As the pH becomes more alkaline, the half-life and reactivity with amines increases; therefore, crosslinking is more efficient when performed at pH 10 than at pH 8. Reaction conditions below pH 10 may result in side reactions, although amidine formation is favored between pH 8-10. Studies using monofunctional alkyl imidates reveal that at pH <10 conjugation can form with just one imidoester functional group. An intermediate N-alkyl imidate forms at the lower pH range and will either crosslink to another amine in the immediate vicinity, resulting in N,N-amidine derivatives, or it will convert to an amidine bond. At higher pH, the amidine is formed directly without formation of an intermediate or side product. Extraneous crosslinking that occurs below pH 10 sometimes interferes with interpretation of results when thiol-cleavable diimidoesters are used.

Although these crosslinkers are still used in protein subunit studies and solid-phase immobilization, the amidine bonds formed by imidoester crosslinkers are reversible at high pH and, therefore, the more stable and efficient NHS-ester crosslinkers have steadily replaced them.

N-Hydroxysuccinimide-esters (NHS-esters)

NHS-esters yield stable products upon reaction with primary amines with relatively efficient coupling at physiological pH. Accessible α-amine groups present on the *N*-termini of proteins and ε-amines on lysine residues react with NHS-esters and form amide bonds. A covalent amide bond is formed when the NHS-ester crosslinking agent reacts with a primary amine, releasing *N*-hydroxysuccinimide (Figure 7).

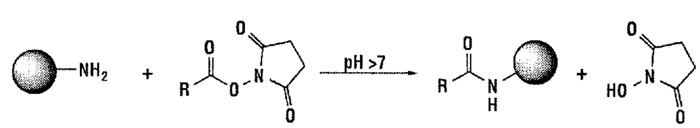


Figure 7. NHS-ester reaction scheme.

Hydrolysis of the NHS-ester competes with the primary amine reaction. Hydrolysis rate increases with increasing pH and occurs more readily in dilute protein solutions. Studies performed on NHS-ester compounds indicate the half-life of hydrolysis for a homobifunctional NHS-ester is 4-5 hours at pH 7.0 and 0°C in aqueous environments free of primary amines. This half-life decreases to 10 minutes at pH 8.6 and 4°C. The extent of the NHS-ester hydrolysis in aqueous solutions free of primary amines may be measured at 260 nm. An increase in absorbance at this wavelength is caused by the release of NHS. The molar extinction coefficient of NHS released by hydrolysis and reaction with a nucleophile is $8.2 \times 10^3\ M^{-1}\ cm^{-1}$ at 260 nm at pH 9.0. The molar extinction coefficient for the NHS-ester in 50 mM potassium phosphate buffer, pH 6.5 is $7.5 \times 10^3\ M^{-1}\ cm^{-1}$ at 260 nm.

NHS-ester crosslinking reactions are most commonly performed in phosphate, bicarbonate/carbonate, HEPES or borate buffers at concentrations between 50-200 mM. Other buffers may also be used if they do not contain primary amines. HEPES, for example, can be used because it contains only tertiary amines. Primary amines are present in the structure of Tris, which makes it an unacceptable buffer for NHS-ester reactions. A large excess of Tris at neutral- to basic-pH may be added at the end of an NHS-ester reaction to quench it. Glycine also contains a primary amine and may be used in a similar manner. The NHS-ester reactions are typically performed between pH 7 and 9 and at 4°C to room temperature from 30 minutes to 2 hours. Reaction times at 4°C are increased four-fold from room temperature incubation times to produce similar efficiencies. NHS-esters are usually used at two- to 50-fold molar excess to protein depending on the concentration of the protein. Typically, the concentration of the crosslinker can vary from 0.1-10 mM. The protein concentration should be kept above 10 µM (50-100 µM is optimal) because more dilute protein solutions result in excessive hydrolysis of the crosslinker.

NHS-esters can be grouped into two separate classes with essentially identical reactivity toward primary amines: water-soluble and water-insoluble. Water-soluble NHS-esters have a sulfonate ($-SO_3^-$) group on the *N*-hydroxysuccinimide ring. They are advantageous when the presence of organic solvents cannot be tolerated. The reaction with the sulfo-NHS-esters is usually performed in 100% aqueous solutions; however, it is possible to achieve greater solubility when the reagent is dissolved in organic solvents such as DMSO (Product # 20686). The water-soluble NHS-ester crosslinkers are used for cell-surface conjugation because they will not permeate the membrane. Sulfonated NHS-ester crosslinkers are supplied as sodium salts and are soluble in water to a concentration of at least 10 mM.

The solubility of the NHS-esters will vary with buffer composition. The non-sulfonated forms of NHS-ester reagents are water-insoluble and are first dissolved in water-miscible organic solvent, such as DMSO (Product # 20684, 20686) and DMF (Product # 20672, 20673), then added to the aqueous reaction mixture. The water-insoluble crosslinkers do not possess a charged group and are lipophilic and membrane-permeable. Crosslinking reactions with the water-insoluble NHS-esters are typically performed with a solvent carryover of 0.5-10% final volume in the aqueous reaction. In some cases, crosslinking proteins with NHS-esters may result in loss of biological activity that may be a result of conformational change of the protein when the NHS-ester crosslinker reacts with primary amines on the molecule's surface. Loss of activity may also occur when any of the lysine groups involved in binding a substrate (in the case of an enzyme) or an antigen (in the case of an antibody) are modified by the crosslinker.

Sulfhydryl-reactive chemistries

Maleimides

Coupling through sulfhydryl groups is advantageous because it can be site-directed, yield cleavable products and allow for sequential coupling. A protein in a complex mixture can be specifically labeled if it is the only one with a free sulfhydryl group on its surface. If there are insufficient quantities of free sulfhydryls, they can be generated by reduction of disulfide bonds. Alternatively, sulfhydryls can be introduced into molecules through reaction with primary amines using 2-Iminothiolane or Traut's Reagent (Product # 26101), SATA (Product # 26102), or SPDP (Product # 21857).

The maleimide group reacts specifically with sulfhydryl groups when the pH of the reaction mixture is between pH 6.5 and 7.5 and forms a stable thioether linkage that is not reversible (Figure 8). At neutral pH, maleimides react with sulfhydryls 1,000-fold faster than with amines, but at pH >8.5, the reaction favors primary amines. Maleimides do not react with tyrosines, histidines or methionines. Hydrolysis of maleimides to a nonreactive maleamic acid can compete with thiol modification, especially above pH 8.0. Thiols must be excluded from reaction buffers used with maleimides because they will compete for coupling sites. Excess maleimides can be quenched at the end of a reaction by adding free thiols. EDTA can be included in the coupling buffer to minimize oxidation of sulfhydryls.

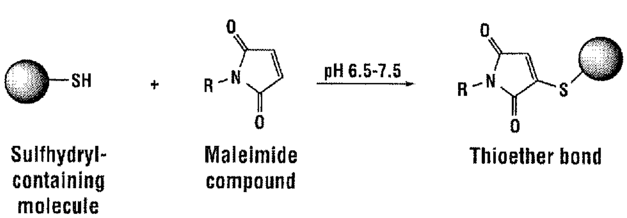


Figure 8. Maleimide reaction scheme.

Haloacetyls

The most commonly used α-haloacetyl crosslinkers contain the iodoacetyl group that reacts with sulfhydryl groups at physiological pH. The reaction of the iodoacetyl group with a sulfhydryl proceeds by nucleophilic substitution of iodine with a thiol producing a stable thioether linkage (Figure 9). Using a slight excess of the iodoacetyl group over the number of sulfhydryl groups at pH 8.3 ensures sulfhydryl selectivity. In the absence of free sulfhydryls, or if a large excess of iodoacetyl group is used, the iodoacetyl group can react with other amino acids. Imidazoles can react with iodoacetyl groups at pH 6.9-7.0, but the incubation must proceed for longer than one week.

Histidyl side chains and amino groups react in the unprotonated form with iodoacetyl groups above pH 5 and pH 7, respectively. To limit free iodine generation, which has the potential to react with tyrosine, histidine and tryptophan residues, perform iodoacetyl reactions and preparations in the dark. Avoid exposure of iodoacetyl compounds to reducing agents. Available NHS-ester haloacetyl crosslinkers are listed in Table 3.

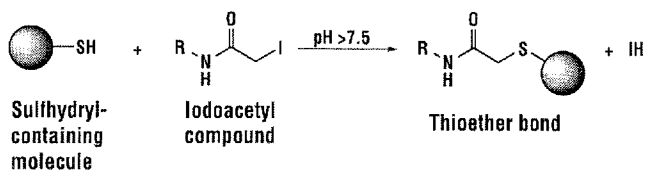


Figure 9. Active halogen reaction scheme.

Histidyl side chains and amino groups react in the unprotonated form with iodoacetyl groups above pH 5 and pH 7, respectively. To limit free iodine generation, which has the potential to react with tyrosine, histidine and tryptophan residues, perform iodoacetyl reactions and preparations in the dark. Avoid exposure of iodoacetyl compounds to reducing agents. Available NHS-ester haloacetyl crosslinkers are listed in Table 3.

Table 3. Available NHS-ester haloacetyl crosslinkers.

Reagent	Reactivity	Product #
SIA	Amine/Sulfhydryl	22349
SIAB	Amine/Sulfhydryl	22329
Sulfo-SIAB	Amine/Sulfhydryl	22327

Pyridyl disulfides

Pyridyl disulfides react with sulfhydryl groups over a broad pH range (optimal pH is 4-5) to form disulfide bonds and, therefore, conjugates prepared using these reagents are cleavable. During the reaction, a disulfide exchange occurs between the molecule's –SH group and the 2-pyridyldithiol group. As a result, pyridine-2-thione is released and its concentration can be determined by measuring the absorbance at 343 nm (Figure 10). These reagents can be used as crosslinkers and to introduce sulfhydryl groups into proteins. The disulfide exchange can be performed at physiological pH, although the reaction rate is slower.
(See Table 4 for the pyridyldithiol crosslinkers available from Pierce.)

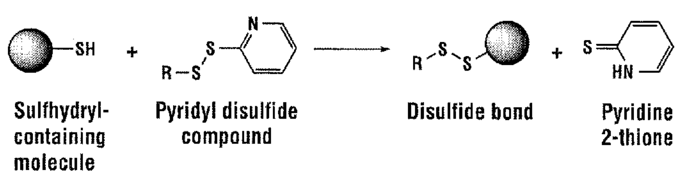


Figure 10. Pyridyl disulfide reaction scheme. Reaction efficiency can be monitored by determining the concentration of the released pyridine-2-thione by measuring the absorbance at 343 nm (molar extinction coefficient at 343 nm = 8.08×10^3 M^{-1} cm^{-1}).

Table 4. Available pyridyl disulfide crosslinkers.

Reagent	Reactivity	Product #
LC-SPDP	Sulfhydryl/Amine	21651
Sulfo-LC-SPDP	Sulfhydryl/Amine	21650
PDPH	Sulfhydryl/Carbohydrate	22301

Carbonyl-/Glyco-reactive chemistry

Hydrazides

Carbonyls (aldehydes and ketones) react with hydrazides and amines at pH 5-7. The reaction with hydrazides is faster than with amines, making them useful for site-specific crosslinking. Carbonyls do not readily exist in proteins; however, mild oxidation of sugar glycols using sodium *meta*-periodate will convert vicinal hydroxyls to aldehydes or ketones (Figure 11). The oxidation is performed in the dark at 0-4°C to prevent side reactions. Subsequent reaction with hydrazides results in formation of a hydrazone bond. Carbohydrate modification is particularly useful for antibodies in which the carbohydrate is located in the Fc region away from binding sites. At 1 mM $NaIO_4$ and a temperature of 0°C, the oxidation is restricted to sialic acid residues. At concentrations of 6-10 mM periodate, other carbohydrates in proteins (including antibodies) will be targeted.

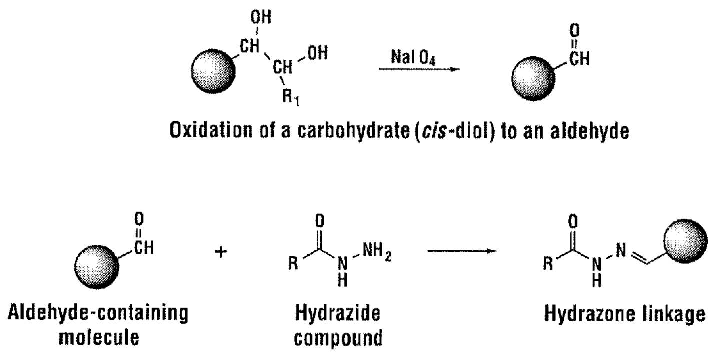


Figure 11. Hydrazide reaction scheme.

Carboxyl-reactive chemistry

Carbodiimides

Carbodiimides couple carboxyls to primary amines or hydrazides, resulting in the formation of amide or hydrazone bonds. Carbodiimides are unlike other conjugation reactions in that no spacer exists between the molecules being coupled. Carboxy termini of proteins can be targeted, as well as glutamic and aspartic acid side chains. In the presence of excess crosslinker, polymerization is likely to occur because all proteins contain carboxyls and amines. The bond that results is the same as a peptide bond, so reversal of the conjugation is impossible without destroying the protein.

EDC (Product # 22980, 22981) reacts with carboxylic acid group and activates the carboxyl group to form an active *O*-acylisourea intermediate, allowing it to be coupled to the amino group in the reaction mixture. An EDC byproduct is released as a soluble urea derivative after displacement by the nucleophile (Figure 12). The *O*-acylisourea intermediate is unstable in aqueous solutions, making it ineffective in two-step conjugation procedures without increasing the stability of the intermediate using *N*-hydroxysuccinimide. This intermediate reacts with a primary amine to form an amide derivative. Failure to react with an amine results in hydrolysis of the intermediate, regeneration of the carboxyls and the release of an *N*-unsubstituted urea. The crosslinking reaction is usually performed between pH 4.5 to 5 and requires only a few minutes for many applications. However, the yield of the reaction is similar at pH from 4.5 to 7.5.

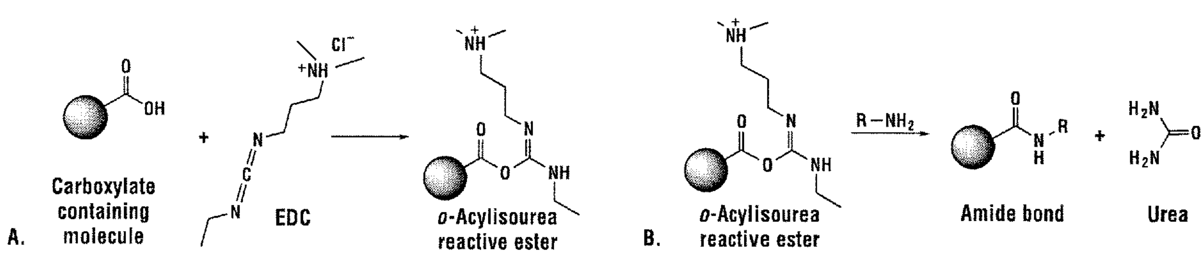


Figure 12. EDC coupling reaction scheme.

Crosslinkers are available in bulk quantities for manufacturing applications

Tel: 800-874-3723 or 815-968-0747 www.piercenet.com/link55d

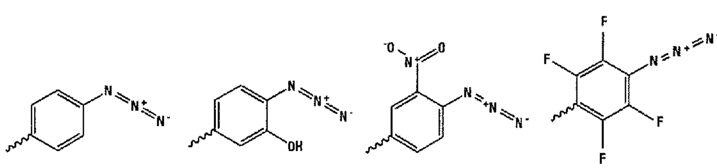
Carbodiimides (continued)

The hydrolysis of EDC is a competing reaction during coupling and is dependent on temperature, pH and buffer composition. 4-Morpholinoethanesulfonic acid (MES, Product # 28390) is an effective carbodiimide reaction buffer. Phosphate buffers reduce the reaction efficiency of the EDC, but increasing the amount of EDC can compensate for the reduced efficiency. Tris, glycine and acetate buffers may not be used as conjugation buffers. NHS (Product # 24500) or its water-soluble analog Sulfo-NHS (Product # 24510) is often included in EDC-coupling protocols to improve efficiency. EDC couples NHS to carboxyls, resulting in an NHS-activated site on a molecule. The NHS-ester formed and the carbodiimide's *O*-acylisourea intermediate are amine-reactive; however, an NHS-ester has much greater stability in slightly acidic or near-neutral pH conditions. In water, an NHS-ester has a half-life of one to several hours, or even days, depending on temperature, pH and structure of the crosslinker, but *O*-acylisourea intermediate has a half-life measured in seconds in acidic or neutral pH conditions. EDC-mediated coupling of molecules works well in many applications without the addition of NHS or Sulfo-NHS, which are not generally required unless protein concentrations are very low. When a large excess of EDC is used without NHS, it is often necessary to reduce the EDC amount when converting to an EDC/NHS system to prevent excessive crosslinking and possible precipitation.

Nonspecific chemistries

Aryl azides

Photoreactive reagents are chemically inert reagents that become reactive when exposed to ultraviolet or visible light. With few exceptions, the photoreactive groups in these reagents are aryl azides (Figure 13). When an aryl azide is exposed to UV light, it forms a nitrene group that can initiate addition reactions with double bonds, insertion into C-H and N-H sites, or subsequent ring expansion to react with a nucleophile (e.g., primary amines, Figure 14). The latter reaction path dominates when primary amines are present in the sample. Thiol-containing reducing agents (e.g., DTT or 2-mercaptoethanol) must be avoided in the sample solution during all steps before and during photoactivation. These reagents will reduce the azide functional group to an amine, preventing photoactivation. Reactions can be performed in a variety of amine-free buffer conditions. If working with hetero-bifunctional photoreactive crosslinkers, use buffers compatible with the chemically reactive portion of the reagent. The chemical reaction is performed in subdued light with reaction vessels covered in foil. The photoactivation can be performed with a bright camera flash or ultraviolet hand-held lamp about one to two inches above the reaction vessels. A bright camera flash works well with the nitro- and hydroxyl-substituted aryl azides. Unsubstituted aryl azides may require ultraviolet light or numerous flashes.



Phenyl Azide | Hydroxyphenyl Azide | Nitrophenyl Azide | Tetrafluorophenyl Azide

Figure 13. Forms of aryl azide-reactive groups in photoreactive crosslinking reagents.

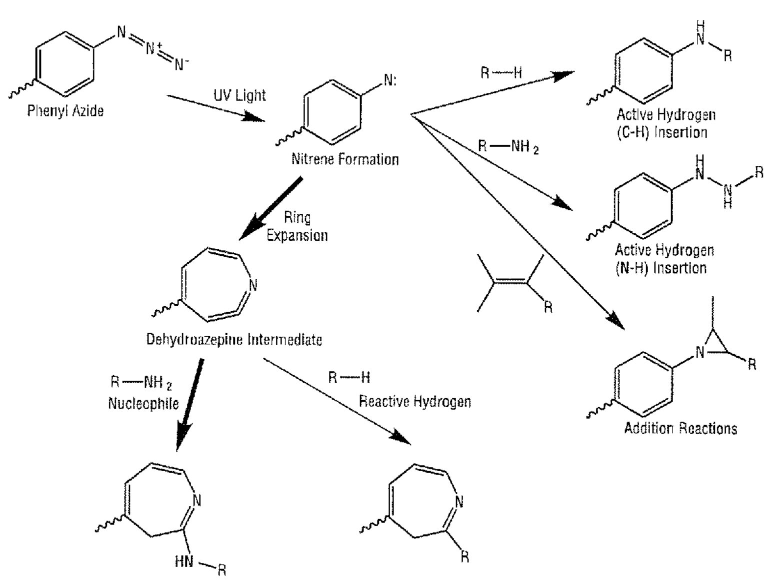


Figure 14. Possible reaction pathways of aryl azide crosslinkers.

Table 5. Available aryl azide crosslinkers.

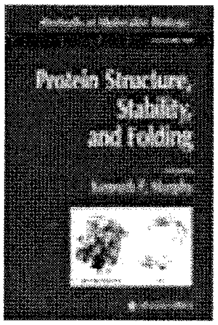
Reagent	Product #	Reactive Groups Photoreactive	Other Groups(s)
ABH	21510	Phenyl azide	Hydrazide
ANB-NOS	21451	Nitrophenyl azide	NHS
APDP	27720	Hydroxyphenyl azide	Pyridyldisulfide
APG	20108	Phenyl azide	Phenylglyoxal
ASBA	21512	Hydroxyphenyl azide	Amine
BASED	21564	Hydroxyphenyl azide	Hydroxyphenyl azide
Mts-Atf-Biotin	33093	Tetrafluorophenyl azide	Methanethiosulfonate/Biotin
Mts-Atf-LC-Biotin	33083	Tetrafluorophenyl azide	Methanethiosulfonate/Biotin
NHS-ASA	27714	Hydroxyphenyl azide	NHS
SADP	21533	Phenyl azide	NHS
SANPAH	22600	Nitrophenyl azide	NHS
SPB	23013	Psoralen	NHS
Sulfo-HSAB	21563	Phenyl azide	Sulfo-NHS
Sulfo-NHS-LC-ASA	27735	Hydroxyphenyl azide	Sulfo-NHS
Sulfo-SADP	21553	Phenyl azide	Sulfo-NHS
Sulfo-SAED	33030	Azido-methylcoumarin	Sulfo-NHS
Sulfo-SAND	21549	Nitrophenyl azide	Sulfo-NHS
Sulfo-SANPAH	22589	Nitrophenyl azide	Sulfo-NHS
Sulfo-SASD	27716	Hydroxyphenyl azide	Sulfo-NHS
Sulfo-SBED	33033	Phenyl azide	Sulfo-NHS/Biotin
Sulfo-SFAD	27719	Perfluoroaryl azide	Sulfo-NHS

Arginine-specific chemistry

Glyoxals are useful compounds for targeting the guanidinyl portion of arginine residues. Glyoxals will target arginines at mildly alkaline pH. There is some cross-reactivity (the greatest at pH ≥9) with lysines. An example of this type of linker is APG (Product # 20108), which has an aryl azide moiety in addition to the glyoxal group. This crosslinker is most useful for targeting compounds deficient in primary amines.

Crosslinkers are available in bulk quantities for manufacturing applications
Tel: 800-874-3723 or 815-968-0747 www.piercenet.com/xlink95d

Books

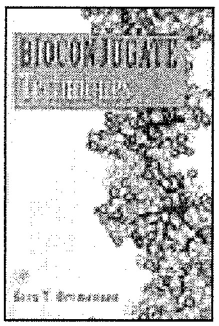


Protein Structure, Stability and Folding

This book covers the methods of calculating stability and dynamics from structural knowledge and for performing molecular dynamics simulations of unfolding. New folding study approaches such as co-solvents, hydrogen exchange and temperature jump are treated along with new strategies for mutagenesis.

Product # 20054 /

Murphy, K.P., Ed., Published by Humana Press, 260 pages, Hardcover

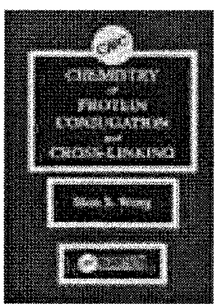


Bioconjugate Techniques

A must for all scientists engaged in protein/immunology research, diagnostics or therapeutics.
An extraordinarily practical look at the major considerations of conjugate formation with user-friendly strategies for constructing bioconjugates of all types.

Product # 20002 /

Hermanson, G.T., Published by Academic Press, Inc., 1995, 785 pages, Softcover

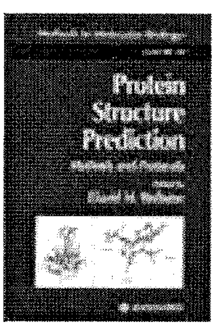


Chemistry of Protein Conjugation and Cross-Linking

An excellent crosslinking reagent reference for the expert and the beginner.
Details the critical aspects of protein crosslinking and conjugate preparation and covers the full spectrum from protein-reactive groups to protein-nucleic acid crosslinking.

Product # 15010 /

Wong, S.S., Ph.D., Published by CRC Press, Inc., 1991, 340 pages, Hardcover

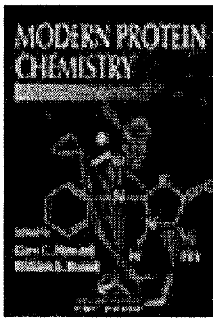


Protein Structure Prediction Methods and Protocol

This important new text is the result of contributions from world-class investigators detailing theory and practice for defining the shape, form and function of proteins. Protocols include sequence alignment, transmembrane protein structure determination, receptor site and active-site prediction as well as identification of motifs and domains and development of suitable folding potentials.

Product # 20048 /

Webster, D.M., Ed., Published by Humana Press, 422 pages, Hardcover

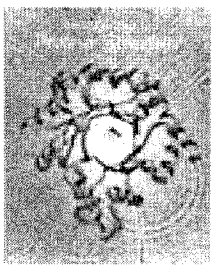


Modern Protein Chemistry — Practical Aspects

This text delves into a focused sampling of new and advanced techniques for studying the structural aspects of proteins. Featured techniques include protein sequencing, mass spectral analysis, NMR methods for structural and functional analysis, computer structure modeling, protein crystallization, and X-ray diffraction methods.

Product # 20059 /

Howard, G.C. and Brown, W.E., Eds., Published by CRC Press, 400 pages, Paperback



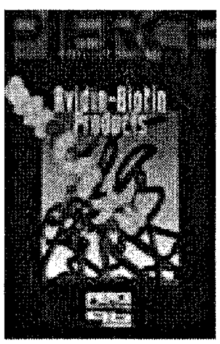
Introduction to Protein Structure

A great primer for understanding protein structure.
Contains 340 illustrations that help explain protein structure.

Product # 20004 /

Branden, C. and Tooze, J., Published by Garland Publishing, Inc., 1991. 302 pages, Softcover

Free technical handbooks!



Avidin-Biotin Products

This reference guide brings together everything needed to biotinylate cell-surface proteins, purify a biotinylated target, detect a biotinylated antibody and perform many other applications. It includes dozens of references along with protocols, troubleshooting tips, selection guides and a complete listing of available tools. Because the Avidin-Biotin system can be used in so many ways, you'll want to keep this booklet close at hand!

Product # 1601321

Buffers for Blotting, ELISA, Histochemistry and Protein Purification Handbook

Improve your assay sensitivity with Pierce blocking buffers. This 20-page handbook provides technical and ordering information that will help you choose the appropriate buffer to maximize signal and minimize background in your application.

Product # 1600702

2

Appendix I

Pierce crosslinkers at a glance

Product #	Abbreviation	Chemical Name	Pkg. Size	M.W.	Spacer Arm
21510	ABH	*p*-Azidobenzoyl hydrazide	50 mg	177.16	11.9 Å
22295	AMAS	*N*-(α-Maleimidoacetoxy)-succinimide ester	50 mg	252.18	4.4 Å
21451	ANB-NOS	*N*-5-Azido-2-nitrobenzyloxy-succinimide	50 mg	305.2	7.7 Å
27720	APDP*	*N*-(4-[*p*-Azidosalicylamido]butyl)-3'-(2'-pyridyldithio) propionamide	50 mg	446.55	21.0 Å
20108	APG****	*p*-Azidophenyl glyoxal monohydrate	50 mg	193.16	9.3 Å
21512	ASBA*	4-(*p*-Azidosalicylamido)-butylamine	50 mg	249.27	16.3 Å
21564	BASED*	*Bis* (β-[4-azidosalicylamido]ethyl) disulfide	50 mg	474.52	21.3 Å
22331	BMB	1,4-*Bis*-Maleimidobutane	50 mg	248.23	10.9 Å
22332	BMDB	1,4-*Bis*-Maleimidyl-2,3-dihydroxybutane	50 mg	280.23	10.2 Å
22330	BMH	*Bis*-Maleimidohexane	50 mg	276.29	16.1 Å
22323	BMOE	*Bis*-Maleimidoethane	50 mg	220.18	8.0 Å
22296	BMPA	*N*-β-Maleimidopropionic acid	100 mg	169.13	5.9 Å
22297	BMPH	*N*-(β-Maleimidopropionic acid)hydrazide•TFA	50 mg	297.19	8.1 Å
22298	BMPS	*N*-(β-Maleimidopropyloxy)succinimide ester	50 mg	266.21	5.9 Å
22336	BM[PEO]$_2$	1,8-*Bis*-Maleimidodiethylene-glycol	50 mg	308.29	14.7 Å
22337	BM[PEO]$_3$	1,11-*Bis*-Maleimidotriethyleneglycol	50 mg	352.34	17.8 Å
21610	BS^{2}G-d$_0$	*Bis* (sulfosuccinimidyl)glutarate-d$_0$	10 mg	530.35	7.7 Å
21615	BS^{2}G-d$_4$	*Bis* (sulfosuccinimidyl)2,2,4,4-glutarate-d$_4$	10 mg	534.38	7.7 Å
21580	BS^3 (Sulfo-DSS)	*Bis* (sulfosuccinimidyl)suberate	50 mg	572.43	11.4 Å
21590	BS^3-d$_0$	*Bis* (sulfosuccinimidyl)suberate-d$_0$	10 mg	572.43	11.4 Å
21595	BS^3-d$_4$	*Bis* (sulfosuccinimidyl)2,2,7,7-suberate-d$_4$	10 mg	576.45	11.4 Å
21581	BS[PEG]$_5$	*Bis* (NHS)PEO$_5$	100 mg	532.5	21.7 Å
21600	BSOCOES	*Bis* (2-[succinimidoxycarbonyloxy]ethyl)sulfone	50 mg	436.35	13 Å
22405	C6-SANH*****	C6-Succinimidyl 4-hydrazinonicotinate acetone hydrazone	25 mg	403.43	14.4 Å
22423	C6-SFB******	C6-Succinimidyl 4-formylbenzoate	25 mg	360.36	13.5 Å
20320	DCC	*N,N*-Dicyclohexylcarbodiimide	100 g	206.33	0 Å
21525	DFDNB	1-5-Difluoro-2,4-dinitrobenzene	50 mg	204.09	3 Å
20660 20663	DMA	Dimethyl adipimidate•2HCl	1 g 50 mg	245.15	8.6 Å
21666 21667	DMP	Dimethyl pimelimidate•2HCl	50 mg 1 g	259.17	9.2 Å
20700	DMS	Dimethyl suberimidate•2HCl	1 g	273.20	11 Å
21702	DPDPB	1,4-Di-(3'-[2'pyridyldithio]propionamido) butane	50 mg	482.71	19.9 Å
20593	DSG	Disuccinimidyl glutarate	50 mg	326.26	7.7 Å
22585	DSP	Dithiobis(succimidylpropionate) (Lomant's Reagent)	1 g	404.42	12 Å

$-NH_2$ Amines	$-SH$ Sulfhydryls	Carbo-hydrates	Nonselective (photoreactive)	$-COOH$ Carboxyls	$-OH$ Hydroxyl	Hetero-bifunctional	Cleavable By
		X	X			X	
X	X					X	
X			X			X	
	X		X			X	Thiols
			X				
			X	X		X	
			X				Thiols
	X						
	X						Periodate
	X						
	X						
X	X					X	
	X	X				X	
X	X					X	
	X						
	X						
X							
X							
X							
X							
X							
X							
X							Base
X							
X							
X				X		X	
X							
X							
X							
X							
	X						Thiols
X							
X							Thiols

Product #	Abbreviation	Chemical Name	Pkg. Size	M.W.	Spacer Arm
21655 21555 21658	DSS	Disuccinimidyl suberate	50 mg 1 g 8 x 2 mg	368.35	11.4 Å
20589	DST	Disuccinimidyl tartarate	50 mg	344.24	6.4 Å
20665	DTBP	Dimethyl 3,3'-dithio*bis*propionimidate•2HC	1 g	309.28	11.9 Å
22335	DTME	Dithio*bis*-maleimidoethane	50 mg	312.37	13.3 Å
21578	DTSSP (Sulfo-DSP)	3,3'-Dithio*bis*(sulfosuccinimidylpropionate)	50 mg	608.51	12 Å
77149 22980 22981	EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride	10 mg 5 g 25 g	191.70	0 Å
21565	EGS	Ethylene glycol *bis*(succinimidylsuccinate)	1 g	456.36	16.1 Å
22306	EMCA	*N*-ε-Maleimidocaproic acid	1 g	211.21	9.4 Å
22106	EMCH	*N*-(ε-Maleimidocaproic acid)hydrazide	50 mg	225.24	11.8 Å
22308	EMCS	*N*-(ε-Maleimidocaproyloxy)succinimide ester	50 mg	308.29	9.4 Å
22309	GMBS	*N*-(γ-Maleimidobutyryloxy)succinimide ester	50 mg	280.23	7.3 Å
22334	HBVS	1,6-Hexane-*bis*-vinylsulfone	50 mg	266.38	14.7 Å
22211	KMUA	*N*-κ-Maleimidoundecanoic acid	100 mg	281.35	15.7 Å
22111	KMUH	*N*-(κ-Maleimidoundecanoic acid)hydrazide	50 mg	295.38	19.0 Å
22362	LC-SMCC	Succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxy-(6-amidocaproate)	50 mg	447.48	16.2 Å
21651	LC-SPDP	Succinimidyl 6-(3'-[2-pyridyl-dithio]propionamido)hexanoate	50 mg	425.52	15.7 Å
22311	MBS	*m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester	50 mg	314.25	7.3 Å
22305	MPBH	4-(4-*N*-Maleimidophenyl)-butyric acid hydrazide•HCl	50 mg	309.75	17.9 Å
33093	Mts-Atf-Biotin**	2-[*N*2-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-*N*6-(6-biotinamidocaproyl)-L-lysinyl]ethylmethanethiosulfate	5 mg	839.95	Mts-Atf 11.1 Å Mts-Biotin 29.3 Å Atf-Biotin 30.7 Å
33083	Mts-Atf-LC-Biotin**	2-{*N*2-[N6-(4-Azido-2,3,5,6-tetrafluorobenzoyl)-*N*6-(6-biotinamidocaproyl)-L-lysinyl]}ethylmethanethiosulfate	5 mg	953.11	Mts-Atf 21.8 Å Mts-Biotin 29.3 Å Atf-Biotin 35.2 Å
27714	NHS-ASA*	*N*-Hydroxysuccinimidyl-4-azidosalicylic acid	50 mg	276.21	8.0 Å
22301	PDPH	3-(2-Pyridyldithio)propionylhydrazide	50 mg	229.32	9.2 Å
28100	PMPI	*N*-(*p*-Maleimidophenyl)isocyanate	50 mg	214.18	8.7 Å
21533	SADP	*N*-Succinimidyl (4'-azidophenyl)1, 3'-dithiopropionate	50 mg	352.39	13.9 Å
22400	SANH*****	Succinimidyl 4-hydrazinonicotinate acetone hydrazone	25 mg	290.27	6.7 Å
22600	SANPAH	*N*-Succinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate	50 mg	390.35	18.2 Å
22339	SBAP	Succinimdyl 3-(bromoacetamido)propionate	50 mg	307.10	6.2 Å
22419	SFB******	Succinimidyl 4-formylbenzoate	100 mg	247.20	5.8 Å
22411	SHTH*****	Succinimidyl 4-hydrazidoterephthalate hydrochloride	25 mg	311.68	7.9 Å
22349	SIA	*N*-succinimidyl iodoacetate	50 mg	283.02	1.5 Å
22329	SIAB	*N*-Succinimidyl(4-iodoacetyl)aminobenzoate	50 mg	402.14	10.6 Å
22360	SMCC	Succinimidyl 4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate	50 mg	334.32	8.3 Å

For more product information, or to download a product instruction booklet, visit *www.piercenet.com/xlink95d.*

$-NH_2$ Amines	$-SH$ Sulfhydryls	Carbo-hydrates	Nonselective (photoreactive)	$-COOH$ Carboxyls	$-OH$ Hydroxyl	Hetero-bifunctional	Cleavable By
X							
X							Periodate
X							Thiols
	X						Thiols
X							Thiols
X				X		X	
X							Hydroxylamine
X	X					X	
	X	X				X	
X	X					X	
X	X					X	
	X						
X	X					X	
	X	X				X	
X	X					X	
X	X					X	Thiols
X	X					X	
	X	X				X	
	X		X				Thiols
	X		X				Thiols
X			X			X	
	X	X				X	Thiols
	X				X	X	
X			X			X	Thiols
X							
X			X			X	
X	X					X	
X							
X							
X	X					X	
X	X					X	
X	X					X	

2

Product #	Abbreviation	Chemical Name	Pkg. Size	M.W.	Spacer Arm
22102	SM[PEG]$_2$	NHS-PEO$_2$-Maliemide	100 mg	425.39	17.6 Å
22104	SM[PEG]$_4$	NHS-PEO$_4$-Maliemide	100 mg	513.5	24.6 Å
22108	SM[PEG]$_8$	NHS-PEO$_8$-Maliemide	100 mg	689.71	39.2 Å
22112	SM[PEG]$_{12}$	NHS-PEO$_{12}$-Maliemide	100 mg	865.92	53.4 Å
22416	SMPB	Succinimidyl 4-(*p*-maleimido-phenyl)butyrate	50 mg	356.33	11.6 Å
22363	SMPH	Succinimidyl-6-(β-maleimidopropionamido)hexanoate	50 mg	379.36	14.2 Å
21558	SMPT	4-Succinimidyloxycarbonyl-methyl-α-(2-pyridyldithio)toluene	50 mg	388.46	20.0 Å
23013	SPB	Succinimidyl-(4-psoralen-8-yloxy)butyrate	50 mg	385.32	8.5-9.5 Å
21857	SPDP	*N*-Succinimidyl 3-(2-pyridyldithio)propionate	50 mg	312.37	6.8 Å
21580	Sulfo-DSS	*See BS^3*			
21566	Sulfo-EGS	Ethylene glycol *bis*(sulfo-succinimidyl succinate)	50 mg	660.45	16.1 Å
22307	Sulfo-EMCS	*N*-(ε-Maleimidocaproyloxy)sulfosuccinimide ester	50 mg	410.33	9.4 Å
22324	Sulfo-GMBS	*N*-(γ-Maleimidobutryloxy)sulfosuccinimide ester	50 mg	382.28	7.3 Å
21563	Sulfo-HSAB	*N*-Hydroxysulfosuccinimidyl-4-azidobenzoate	50 mg	362.25	9.0 Å
21111	Sulfo-KMUS	*N*-(κ-Maleimidoundecanoyloxy)sulfosuccinimide ester	50 mg	485.47	16.3 Å
21568	Sulfo-LC-SMPT	Sulfosuccinimidyl 6-(α-methyl-α-[2-pyridyldithio]-toluamido)hexanoate	50 mg	603.67	20.0 Å
21650	Sulfo-LC-SPDP	Sulfosuccinimidyl 6-(3'-[2-pyridyl-dithio]propionamido)hexanoate	50 mg	527.57	15.7 Å
22312	Sulfo-MBS	*m*-Maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester	50 mg	416.30	7.3 Å
27735	Sulfo-NHS-LC-ASA*	Sulfosuccinimidyl(4-azido-salicylamido)hexanoate	50 mg	491.41	18.0 Å
21553	Sulfo-SADP	Sulfosuccinimidyl(4-azido-phenyldithio)propionate	50 mg	454.44	13.9 Å
33030	Sulfo-SAED	Sulfosuccimidyl 2-[7-azido-4-methylcoumarin-3-acetamido]ethyl-1,3'-dithiopropionate	5 mg	621.6	23.6 Å
21549	Sulfo-SAND	Sulfosuccinimidyl-2-(*m*-azido-*o*-nitrobenzamido) ethyl 1,3'-dithiopropionate	50 mg	570.51	18.5Å
22589	Sulfo-SANPAH	Sulfosuccinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate	50 mg	492.40	18.2 Å
27716	Sulfo-SASD*	Sulfosuccinimidyl 2-(*p*-azido-salicylamido)ethyl 1,3'-dithiopropionate	50 mg	541.51	18.9 Å
33033	Sulfo-SBED**	Sulfo-NHS-(2-6-[Biotinamido]-2-(*p*-azidobezamido) hexanoamido)ethyl-1,3'-dithiopropionate *(Trifunctional)*	10 mg	879.98	Sulfo-NHS ester 13.7 Å Phenyl azide 9.1 Å Biotin 19.1 Å
27719	Sulfo-SFAD	Sulfosuccinimidyl(perfluoroazidobenzamido) ethyl 1,3'-dithiopropionate	50 mg	597.48	14.6 Å
22327	Sulfo-SIAB	Sulfosuccinimidyl(4-iodo-acetyl)aminobenzoate	50 mg	504.19	10.6 Å
22322	Sulfo-SMCC	Sulfosuccinimidyl 4-(*N*-maleimido-methyl)cyclohexane-1-carboxylate	50 mg	436.37	8.3 Å
22317	Sulfo-SMPB	Sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate	50 mg	458.38	11.6 Å
22607	THPP	β-(Tris[hydroxymethyl]phosphine)propionic acid (betaine)	50 mg	197.15	3.03 Å
33043	TMEA***	*Tris*-(2-Maleimidoethyl)amine *(Trifunctional)*	50 mg	386.36	10.3 Å
33063	TSAT***	*Tris*-(succimimidyl aminotricetate) *(Trifunctional)*	50 mg	482.36	4.2 Å

* *Crosslinker is iodinatable.*
** *Trifunctional crosslinking reagent; binds to Avidin, Streptavidin or NeutrAvidin™ Protein.*
*** *Trifunctional crosslinking reagent.*
**** *Reacts selectively with arginine at pH 7-8.*
***** *Carbonyl reactive.*
****** *Hydrazine/Hydrazone reactive.*

$-NH_2$ Amines	$-SH$ Sulfhydryls	Carbo-hydrates	Nonselective (photoreactive)	$-COOH$ Carboxyls	$-OH$ Hydroxyl	Hetero-bifunctional	Cleavable By
X	X					X	
X	X					X	
X	X					X	
X	X					X	
X	X					X	
X	X					X	Thiols
X	X					X	
X			X			X	
X	X					X	Thiols
X							Hydroxylamine
X	X					X	
X	X					X	
X			X			X	
X	X					X	
X	X					X	Thiols
X	X					X	Thiols
X	X					X	
X			X			X	
X			X			X	Thiols
X			X			X	Thiols
X			X			X	Thiols
X			X			X	
X			X			X	Thiols
	X			X		X	Thiols
X			X			X	Thiols
X	X					X	
X	X					X	
X	X					X	
X							
	X						
X							

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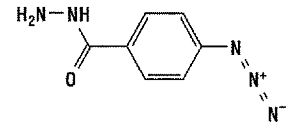
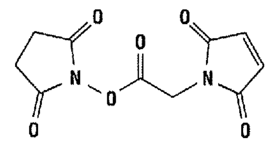
Appendix II

Structures (see Appendix III for references cited after abbreviations)

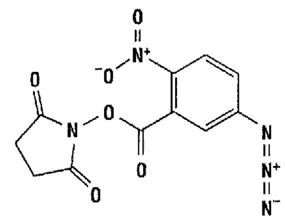
ABH
M.W. 177.16
Spacer Arm 11.9 Å



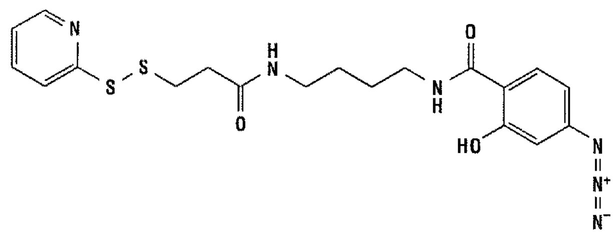
AMAS[57,58]
M.W. 252.18
Spacer Arm 4.4 Å



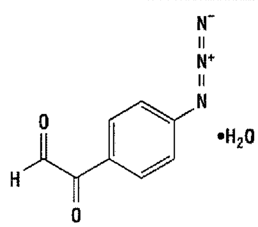
ANB-NOS[2]
M.W. 305.20
Spacer Arm 7.7 Å



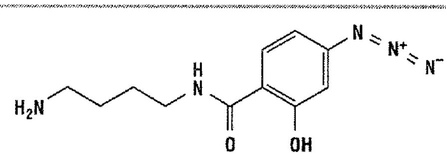
APDP[3]
M.W. 446.55
Spacer Arm 21.0 Å



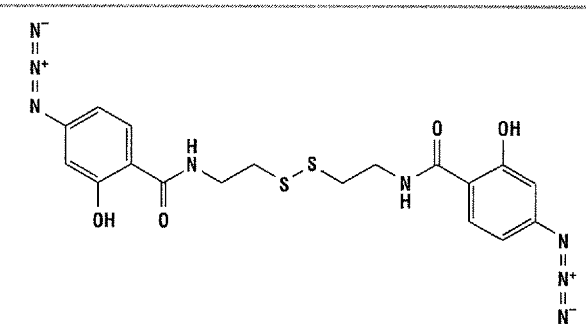
APG[4]
M.W. 193.16
Spacer Arm 9.3 Å



ASBA[5]
M.W. 249.27
Spacer Arm 16.3 Å

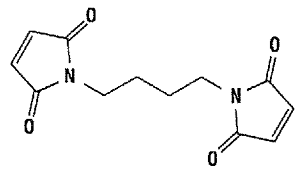


BASED[6]
M.W. 474.52
Spacer Arm 21.3 Å

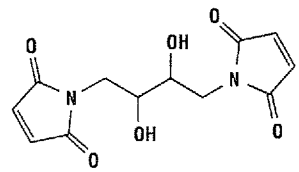


For more product information, or to download a product instruction booklet, visit www.piercenet.com/xlink95d.

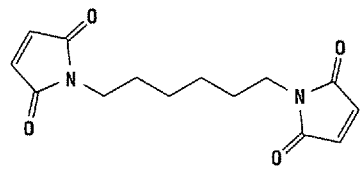
BMB[7]
M.W. 248.23
Spacer Arm 10.9 Å



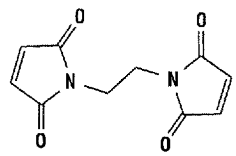
BMDB[59-62]
M.W. 280.23
Spacer Arm 10.2 Å



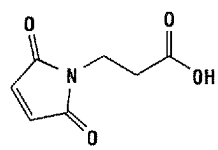
BMH[7]
M.W. 276.29
Spacer Arm 16.1 Å



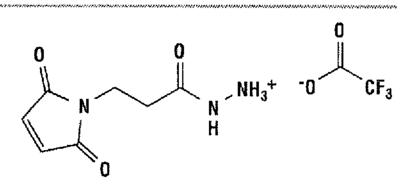
BMOE[7]
M.W. 220.18
Spacer Arm 8.0 Å



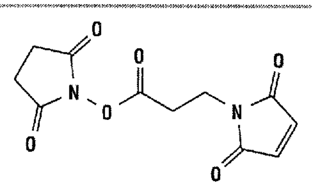
BMPA[5,63,64]
M.W. 169.13
Spacer Arm 5.9 Å



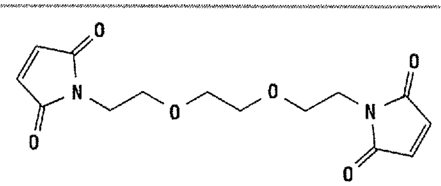
BMPH[2,84]
M.W. 297.19
Spacer Arm 8.1 Å



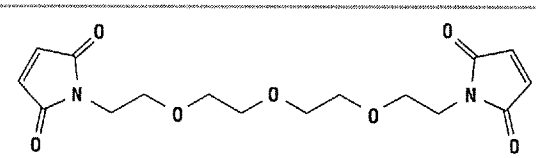
BMPS[32,43]
M.W. 266.21
Spacer Arm 5.9 Å



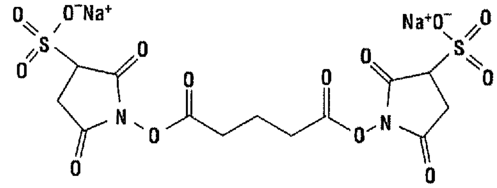
BM[PEO]$_2$
M.W. 308.29
Spacer Arm 14.7 Å



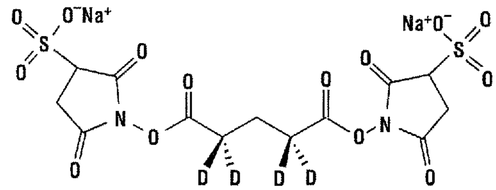
BM[PEO]$_3$
M.W. 352.34
Spacer Arm 17.8 Å



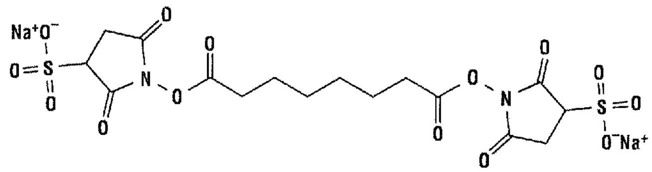
BS^2G-d_0
M.W. 530.35
Spacer Arm 7.7 Å



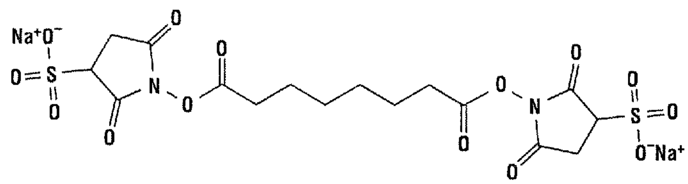
BS^2G-d_4
M.W. 534.38
Spacer Arm 7.7 Å



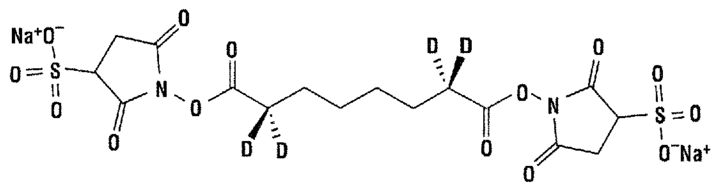
BS^3 (Sulfo-DSS)[9]
M.W. 572.43
Spacer Arm 11.4 Å



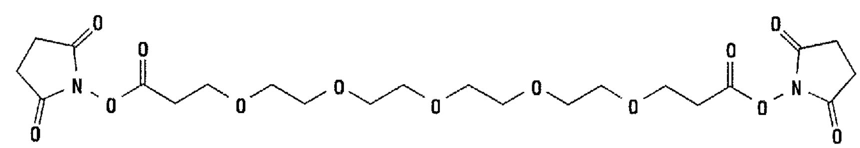
BS^3-d_0
M.W. 572.43
Spacer Arm 11.4 Å



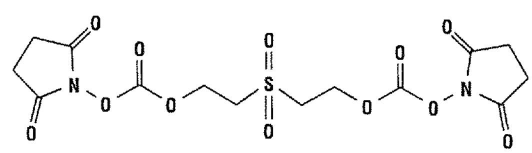
BS^3-d_4
M.W. 576.45
Spacer Arm 11.4 Å



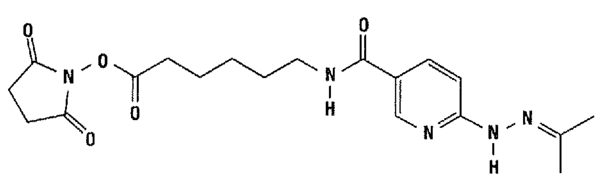
$BS[PEG]_5$
M.W. 532.5
Spacer Arm 21.7 Å



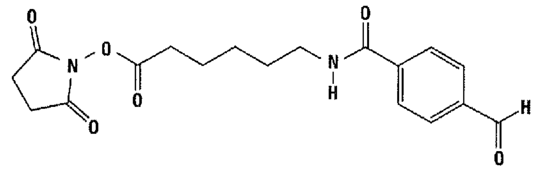
BSOCOES[8,39]
M.W. 436.35
Spacer Arm 13.0 Å



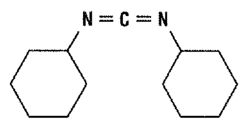
C6-SANH
M.W. 403.43
Spacer Arm 14.4 Å



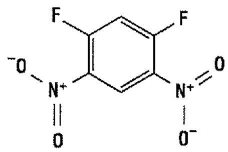
C6-SFB
M.W. 360.36
Spacer Arm 13.5 Å



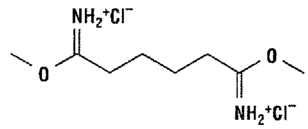
DCC
M.W. 206.33



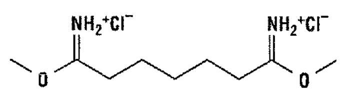
DFDNB [10]
M.W. 204.09
Spacer Arm 3.0 Å



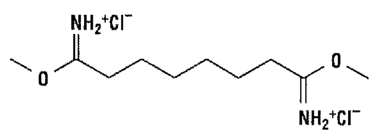
DMA [11]
M.W. 245.15
Spacer Arm 8.6 Å



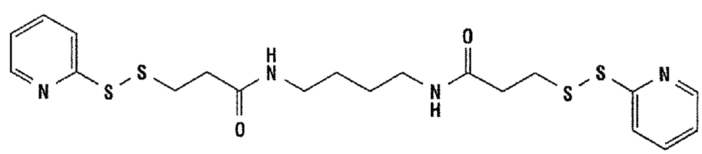
DMP [12]
M.W. 259.17
Spacer Arm 9.2 Å



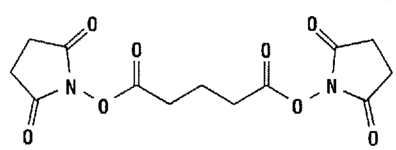
DMS [13]
M.W. 273.20
Spacer Arm 11.0 Å



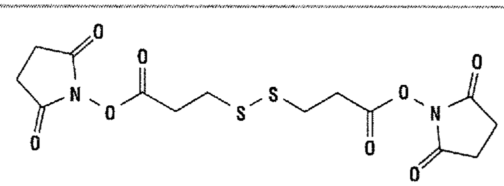
DPDPB [14]
M.W. 482.71
Spacer Arm 19.9 Å



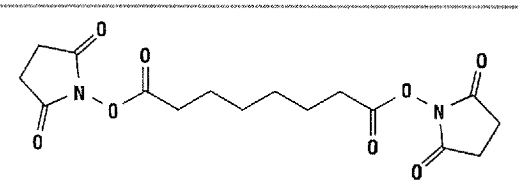
DSG [15]
M.W. 326.26
Spacer Arm 7.7 Å



DSP [16]
M.W. 404.42
Spacer Arm 12.0 Å



DSS [17]
M.W. 368.35
Spacer Arm 11.4 Å



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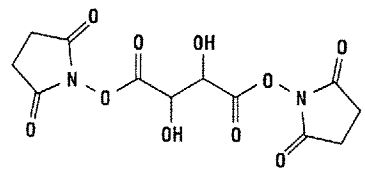
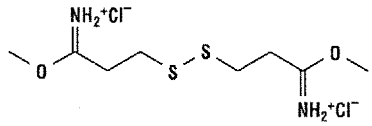
Structures [continued]

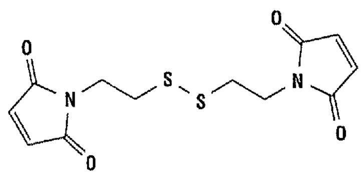
DST[18,40]
M.W. 344.24
Spacer Arm 6.4 Å



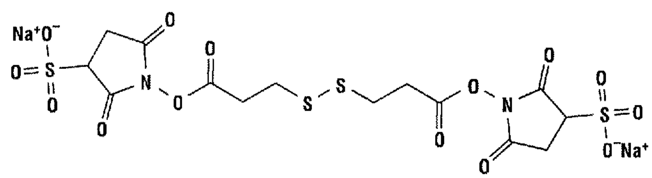
DTBP[19]
M.W. 309.28
Spacer Arm 11.9 Å



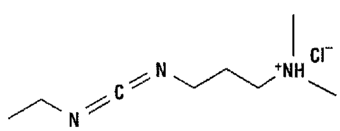
DTME[7,65]
M.W. 312.37
Spacer Arm 13.3 Å



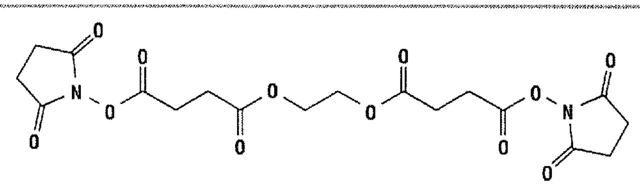
DTSSP (Sulfo-DSP)[20]
M.W. 608.51
Spacer Arm 12.0 Å



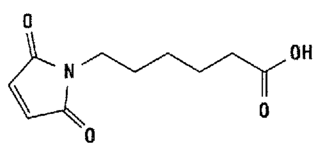
EDC[21,88]
M.W. 191.70
Spacer Arm 0 Å



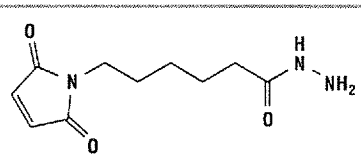
EGS[22,41]
M.W. 456.36
Spacer Arm 16.1 Å



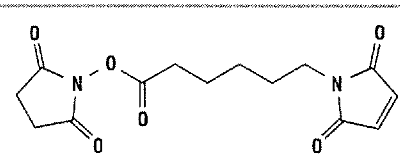
EMCA[66]
M.W. 211.21
Spacer Arm 9.4 Å



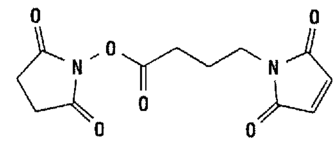
EMCH[67]
M.W. 225.24
Spacer Arm 11.8 Å



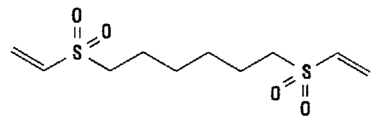
EMCS[23,68,69]
M.W. 308.29
Spacer Arm 9.4 Å



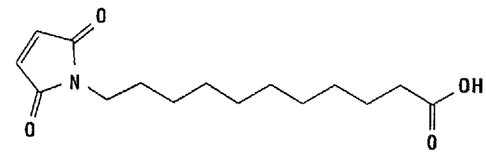
GMBS[23,84]
M.W. 280.23
Spacer Arm 7.3 Å



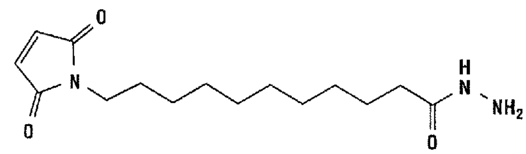
HBVS[78-80]
M.W. 266.38
Spacer Arm 14.7 Å



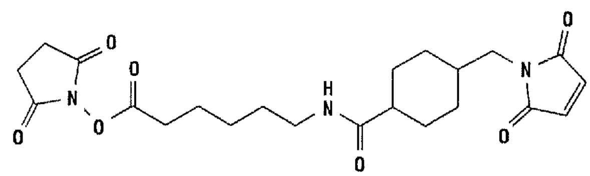
KMUA[63-64]
M.W. 281.35
Spacer Arm 15.7 Å



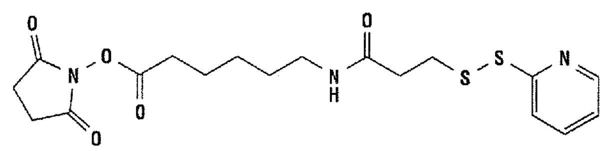
KMUH[67]
M.W. 295.38
Spacer Arm 19.0 Å



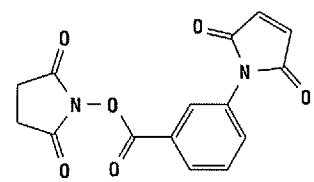
LC-SMCC[35,52,70]
M.W. 447.48
Spacer Arm 16.2 Å



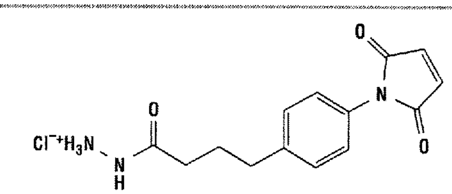
LC-SPDP[33,38]
M.W. 425.52
Spacer Arm 15.7 Å



MBS[25,42,84]
M.W. 314.25
Spacer Arm 7.3 Å



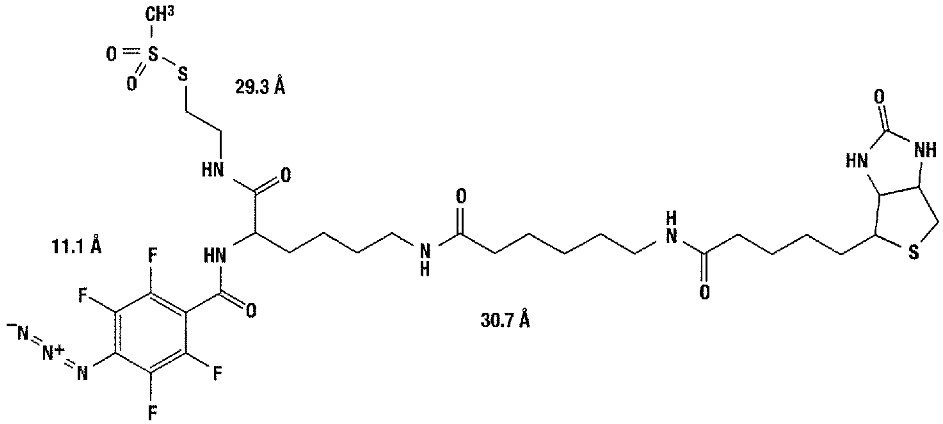
MPBH[26]
M.W. 309.75
Spacer Arm 17.9 Å



Mts-Atf-Biotin

M.W. 839.95
Spacer Arms

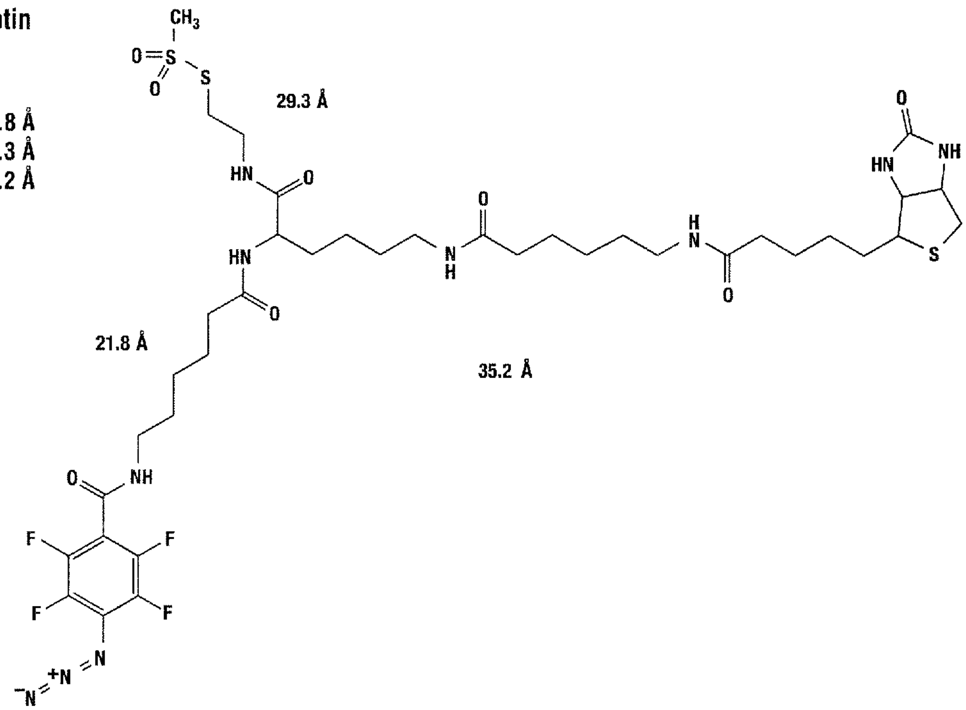
Mts-Atf	11.1 Å
Mts-Biotin	29.3 Å
Atf-Biotin	30.7 Å



Mts-Atf-LC-Biotin

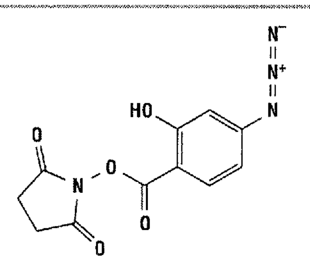
M.W. 953.11
Spacer Arms

Mts-Atf	21.8 Å
Mts-Biotin	29.3 Å
Atf-Biotin	35.2 Å



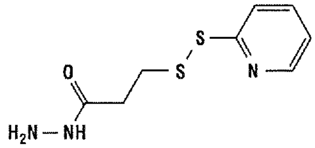
NHS-ASA[27]

M.W. 276.21
Spacer Arm 8.0 Å

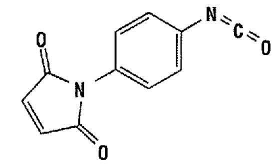


PDPH[28,76,77]

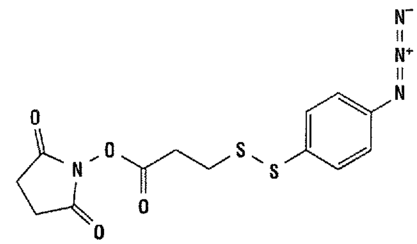
M.W. 229.32
Spacer Arm 9.2 Å



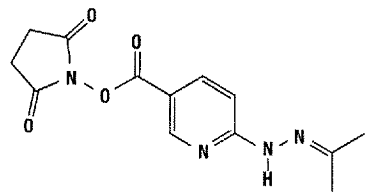
PMPI[71]
M.W. 214.18
Spacer Arm 8.7 Å



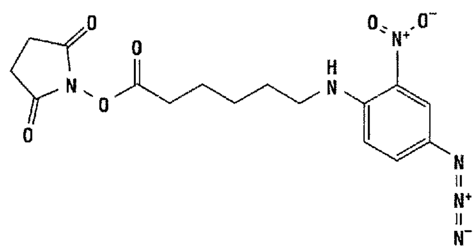
SADP[53]
M.W. 352.29
Spacer Arm 13.9 Å



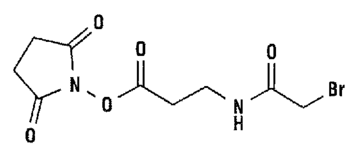
SANH
M.W. 290.27
Spacer Arm 6.7 Å



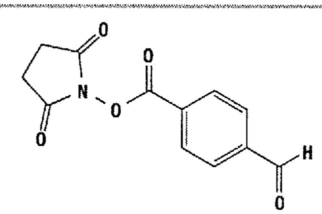
SANPAH[30]
M.W. 390.35
Spacer Arm 18.2 Å



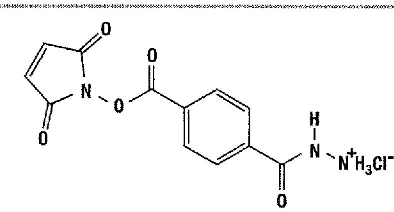
SBAP[73]
M.W. 307.10
Spacer Arm 6.2 Å



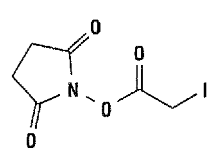
SFB
M.W. 247.20
Spacer Arm 5.8 Å



SHTH
M.W. 311.68
Spacer Arm 7.9 Å



SIA[74,75]
M.W. 283.02
Spacer Arm 1.5 Å



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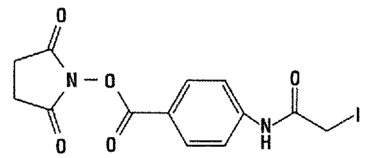
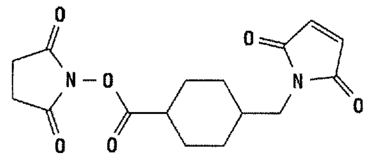
Structures [continued]

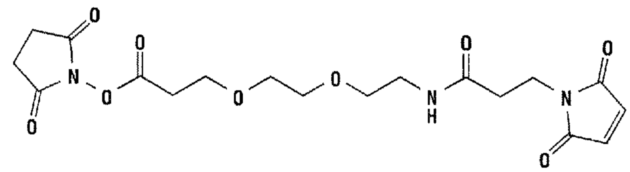
SIAB[33,34]
M.W. 402.14
Spacer Arm 10.6 Å



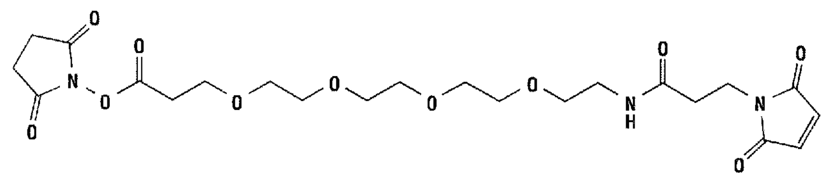
SMCC
M.W. 334.32
Spacer Arm 8.3 Å



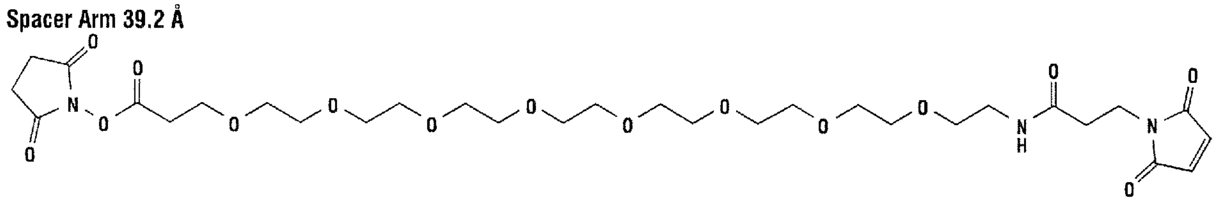
SM[PEG]$_2$
M.W. 425.39
Spacer Arm 17.6 Å



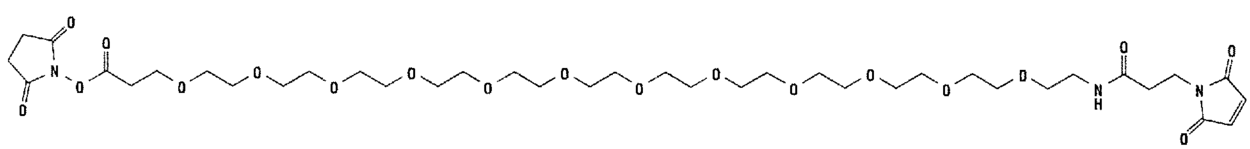
SM[PEG]$_4$
M.W. 513.5
Spacer Arm 24.6 Å



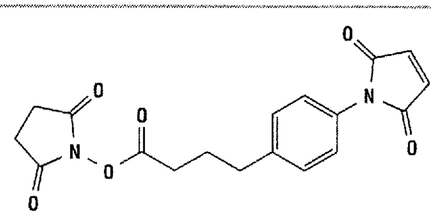
SM[PEG]$_8$
M.W.689.71
Spacer Arm 39.2 Å



SM[PEG]$_{12}$
M.W. 865.92
Spacer Arm 53.4 Å



SMPB
M.W. 356.33
Spacer Arm 11.6 Å



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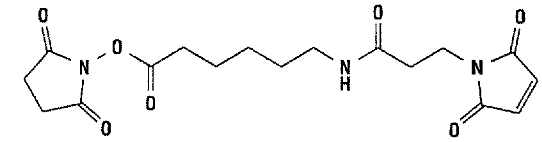
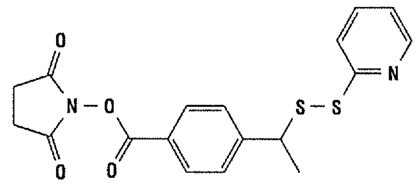
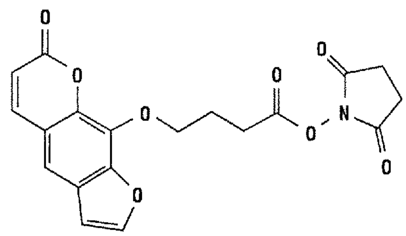
SMPH[74,75]
M.W. 379.36
Spacer Arm 14.2 Å



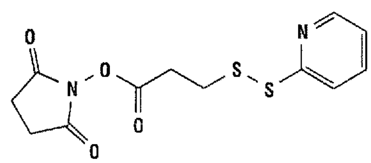
SMPT[37]
M.W. 388.46
Spacer Arm 20.0 Å



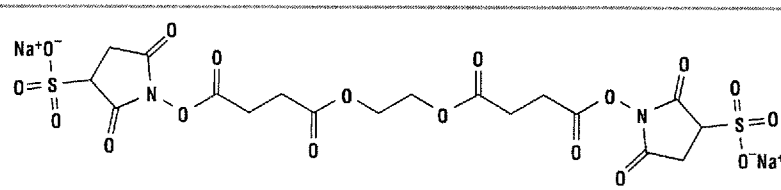
SPB[97-99]
M.W. 385.32
Spacer Arm 8.6 Å



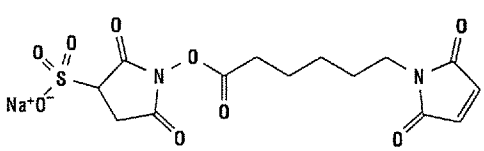
SPDP[38,86]
M.W. 312.37
Spacer Arm 6.8 Å



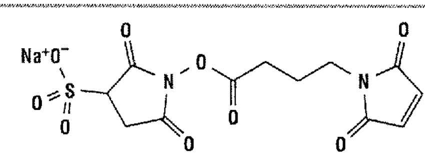
Sulfo-EGS[41]
M.W. 660.45
Spacer Arm 16.1 Å



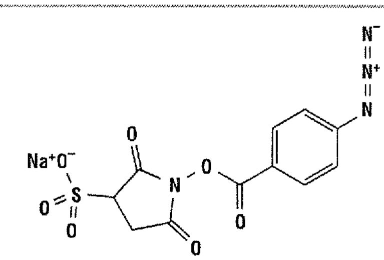
Sulfo-EMCS[23,68,69]
M.W. 410.33
Spacer Arm 9.4 Å



Sulfo-GMBS[23]
M.W. 382.28
Spacer Arm 7.3 Å

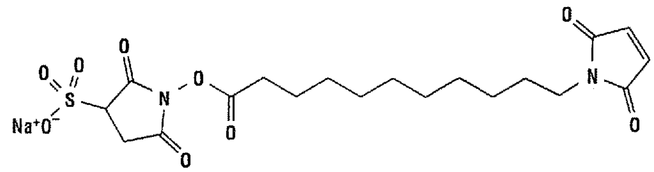


Sulfo-HSAB
M.W. 362.25
Spacer Arm 9.0 Å

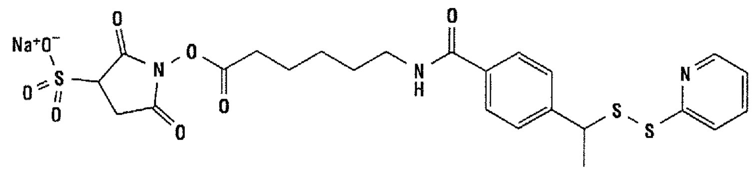


Structures [continued]

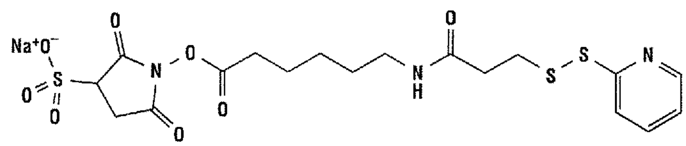
Sulfo-KMUS[23]
M.W. 480.47
Spacer Arm 16.3 Å



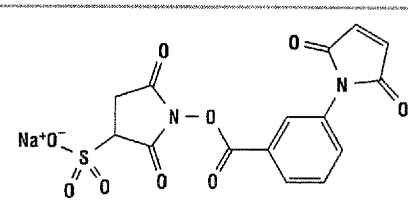
Sulfo-LC-SMPT[48]
M.W. 603.67
Spacer Arm 20.0 Å



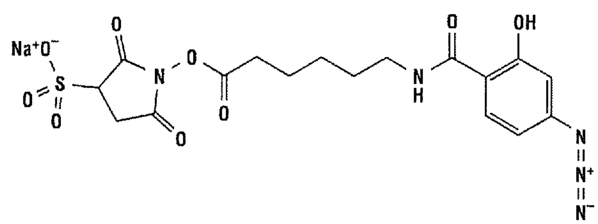
Sulfo-LC-SPDP[38,85]
M.W. 527.57
Spacer Arm 15.7 Å



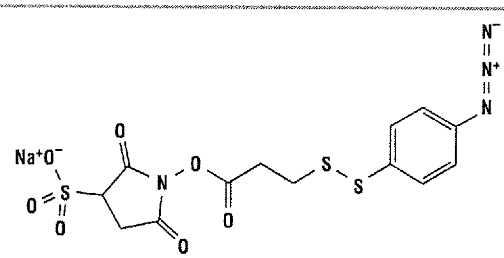
Sulfo-MBS[42]
M.W. 416.30
Spacer Arm 7.3 Å



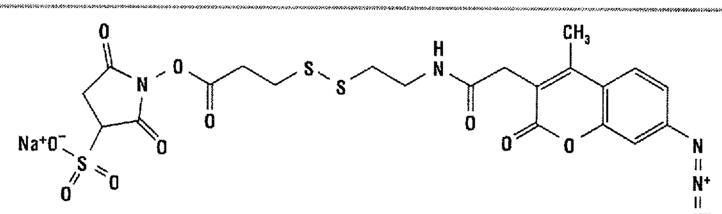
Sulfo-NHS-LC-ASA[27]
M.W. 491.41
Spacer Arm 18.0 Å



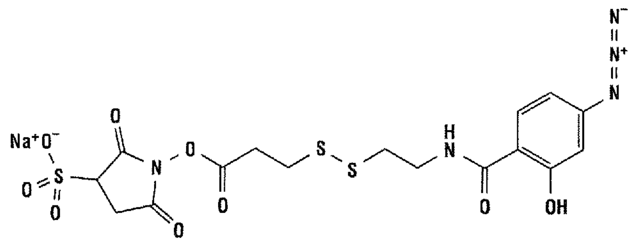
Sulfo-SADP[20]
M.W. 454.44
Spacer Arm 13.9 Å



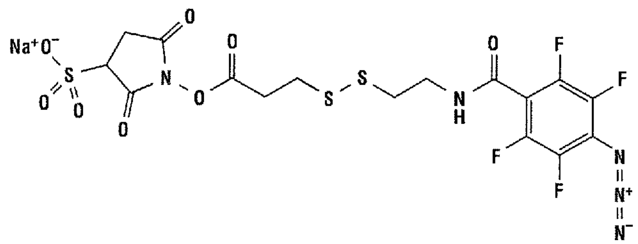
Sulfo-SAED[54]
M.W. 621.60
Spacer Arm 23.6 Å



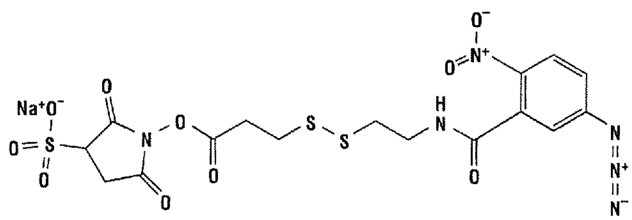
Sulfo-SASD[31,51]
M.W. 541.51
Spacer Arm 18.9 Å



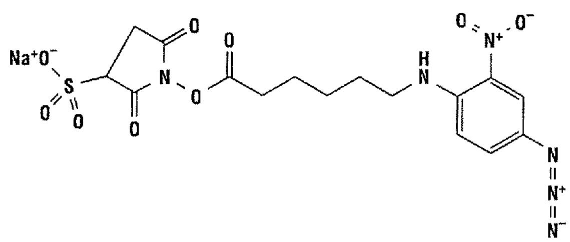
Sulfo-SFAD[83]
M.W. 597.48
Spacer Arm 14.6 Å



Sulfo-SAND[29]
M.W. 570.51
Spacer Arm 18.5 Å

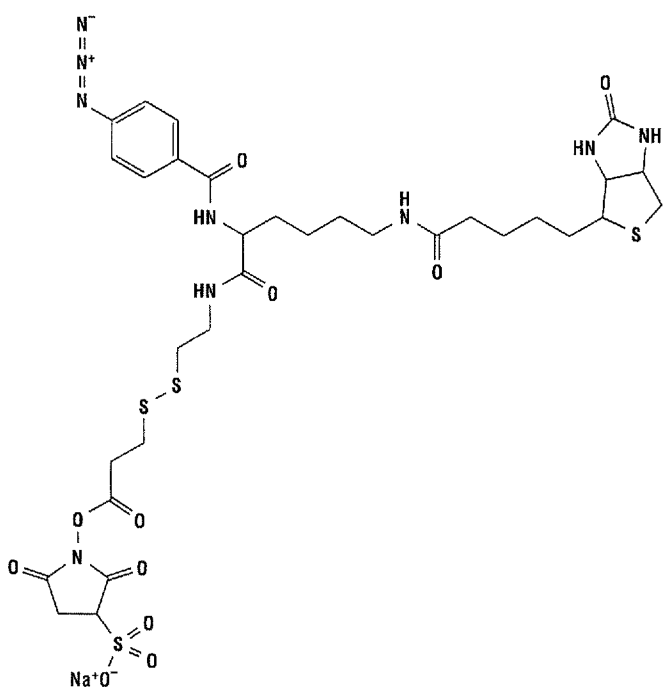


Sulfo-SANPAH[30]
M.W. 492.40
Spacer Arm 18.2 Å

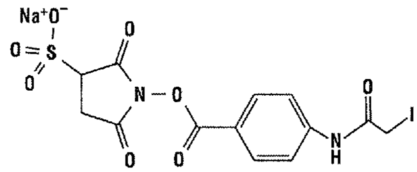


Sulfo-SBED[49,50,87,91,100-106]
M.W. 879.98
Spacer Arms

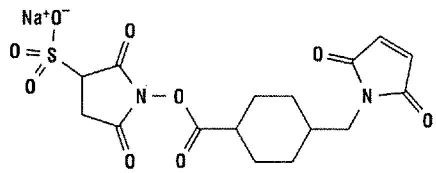
Sulfo-NHS ester	13.7 Å
Phenyl azide	9.1 Å
Biotin	19.1 Å



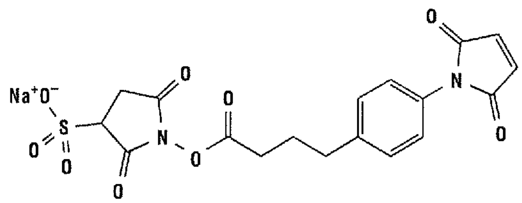
Sulfo-SIAB[45]
M.W. 504.19
Spacer Arm 10.6 Å



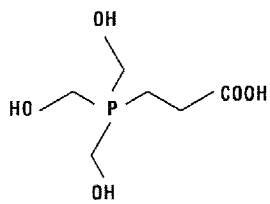
Sulfo-SMCC[46]
M.W. 436.37
Spacer Arm 8.3 Å



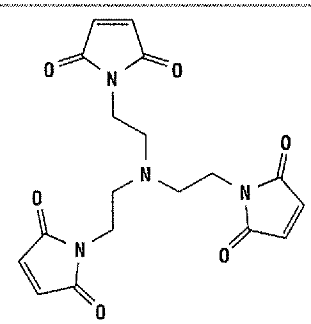
Sulfo-SMPB[36,47]
M.W. 458.38
Spacer Arm 11.6 Å



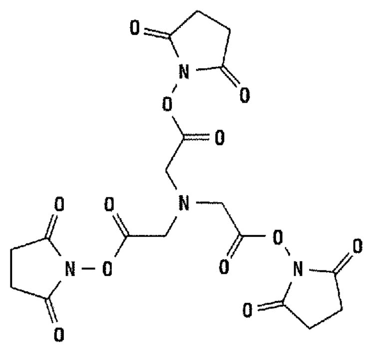
THPP[92-96]
M.W. 197.15
Spacer Arm 3.03 Å



TMEA
M.W. 386.36
Spacer Arm 10.3 Å



TSAT
M.W. 482.36
Spacer Arm 4.2 Å



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Appendix III

Pierce Crosslinking Reagent Literature References

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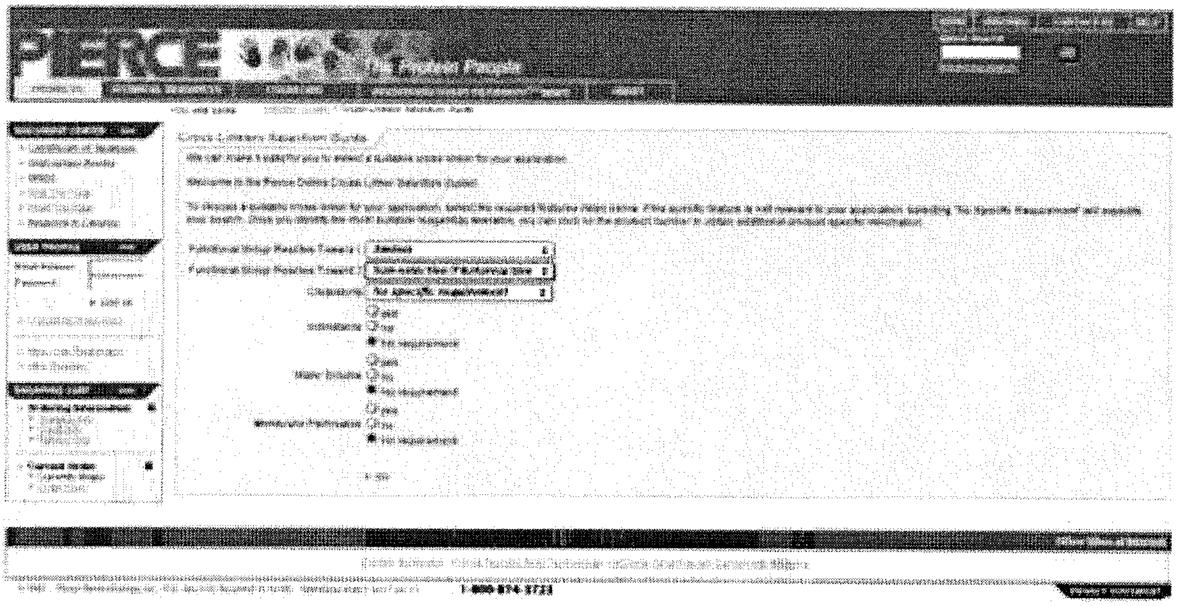
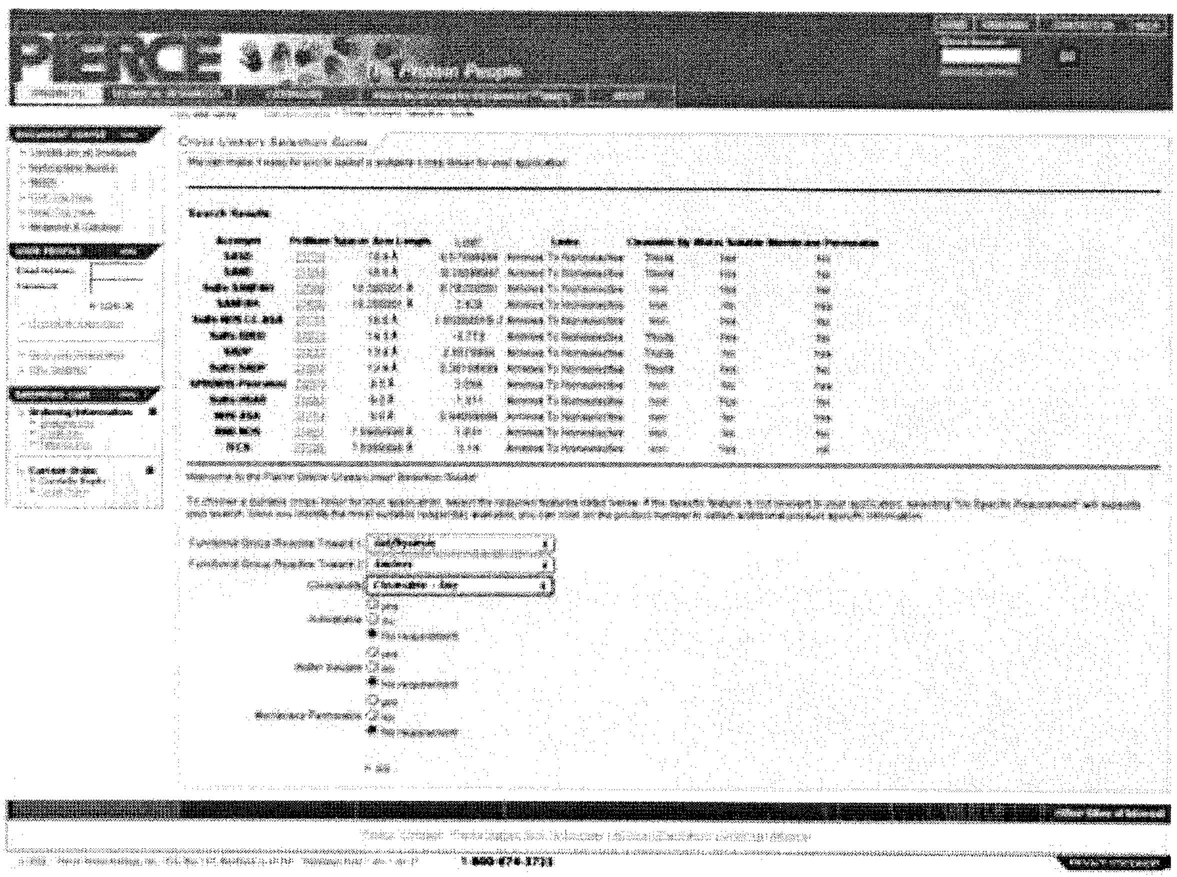
Appendix IV

Online interactive crosslinker selection guide

Pierce has developed an interactive crosslinker selection guide to aid in deciding which crosslinker is the best for your application. Go to *www.piercenet.com*, choose "selection guides" from the Products drop-down menu and then choose the crosslinker selection guide. The interactive selection guide will guide you through the process of choosing the appropriate crosslinker for your application.





Appendix V

Glossary of crosslinking terms

Acylation: Reaction that introduces an acyl group (-COR) into a compound.

Aryl azide: Compound containing a photoreactive functional group (e.g., phenyl azide) that reacts nonspecifically with target molecules.

Carbodiimide: Reagent that catalyzes the formation of an amide linkage between a carboxyl (–COOH) group and a primary amine ($-NH_2$) or a hydrazide ($-NHNH_2$). These reagents do not result in the formation of a cross-bridge and have been termed zerolength crosslinkers.

Crosslinker: A reagent that will react with functional groups on two or more molecules to form a covalent linkage between the molecules.

Conjugation reagent: A crosslinker or other reagent for covalently linking two molecules.

Disulfide bonds: Oxidized form of sulfhydryls (- S – S -); formed in proteins through – SH groups from two cysteine molecules. These bonds often link polypeptide chains together within the protein and contribute to a protein's tertiary structure.

α-Haloacyl: Functional group (e.g., iodoacetyl) that targets nucleophiles, especially thiols. α-Haloacyl compounds have a halogen atom such as iodine, chlorine or bromine attached to an acyl group on the molecule. These alkylating reagents degrade when exposed to direct light or reducing agents, resulting in the loss of the halogen and the appearance of a characteristic color.

Hapten: A molecule recognized by antibodies but unable to elicit an immune response unless attached to a carrier protein. Haptens are usually, but not always, small (< 5 kDa) molecules.

Homobifunctional crosslinker: Reagent with two identical reactive groups used to link two molecules or moieties.

Heterobifunctional crosslinker: Reagent with two different reactive groups used to link two molecules or moieties.

Hydrophilic: Substances that readily dissolve in water.

Hydrophobic: Substances with limited solubility in water.

***N*-Hydroxysuccinimidyl (NHS) ester:** Acylating reagents commonly used for crosslinking or modifying proteins. They are specific for primary ($-NH_2$) amines between pH 7-9, but are generally the most effective at neutral pH. These esters are subject to hydrolysis, with half-lives approximating one to two hours at room temperature at neutral pH.

Imidate crosslinker: Primary amine-reactive functional group that forms an amidine bond. The ε-amine in lysine and N-terminal amines are the targets in proteins. Imidates react with amines in alkaline pH conditions (pH range 7.5-10) and hydrolyze quickly, with half-lives typically around 10-15 minutes at room temperature and pH 7-9. At pH > 11, the amidine bond is unstable, and crosslinking can be reversed. The amidine bond is protonated at physiological pH; therefore, it carries a positive charge.

Imidoester: Amine-reactive functional group of an imidate crosslinker.

Immunogen: A substance capable of eliciting an immune response.

Integral membrane protein: Protein that extends through the cell membrane and is stabilized by hydrophobic interactions within the lipid bilayer of the membrane.

Ligand: A molecule that binds specifically to another molecule. For example, a protein that binds to a receptor.

Moiety: An indefinite part of a sample or molecule.

Monomer: Consisting of a single unit.

NHS: Abbreviation for *N*-hydroxysuccinimide.

Nitrene: Triple-bonded nitrogen-to-nitrogen reactive group formed after exposure of an azido group to UV light. Its reactivity is nonspecific and short-lived.

Nonselective crosslinking: Crosslinking using a reactive group, such as nitrenes or aryl azides, which react so quickly and broadly that specific groups are not easily and efficiently targeted. Yields are generally low with many different crosslinked products formed.

Nonspecific crosslinking: Another term for nonselective crosslinking.

Oligomer: A molecule composed of several monomers.

Photoreactive: A functional group that becomes reactive upon excitation with light at a particular range of wavelengths.

Polymer: A molecule composed of many repeating monomers.

Pyridyl disulfide: Aromatic moiety with a disulfide attached to one of the carbons adjacent to the nitrogen in a pyridine ring. Pyridine 2-thione is released when this reagent reacts with a sulfhydryl (–SH)-containing compound.

Spacer arm: The part of a crosslinker that is incorporated between two crosslinked molecules and serves as a bridge between the molecules.

Substrate: A substance upon which an enzyme acts.

Sulfhydryl: –SH groups present on cysteine residues in proteins.

Thiols: Also known as mercaptens, thiolanes, sulfhydryls or –SH groups, these are good nucleophiles that may be targeted for crosslinking.

Ultraviolet: Electromagnetic radiation of wavelengths between 10-390 nm.

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The Protein People

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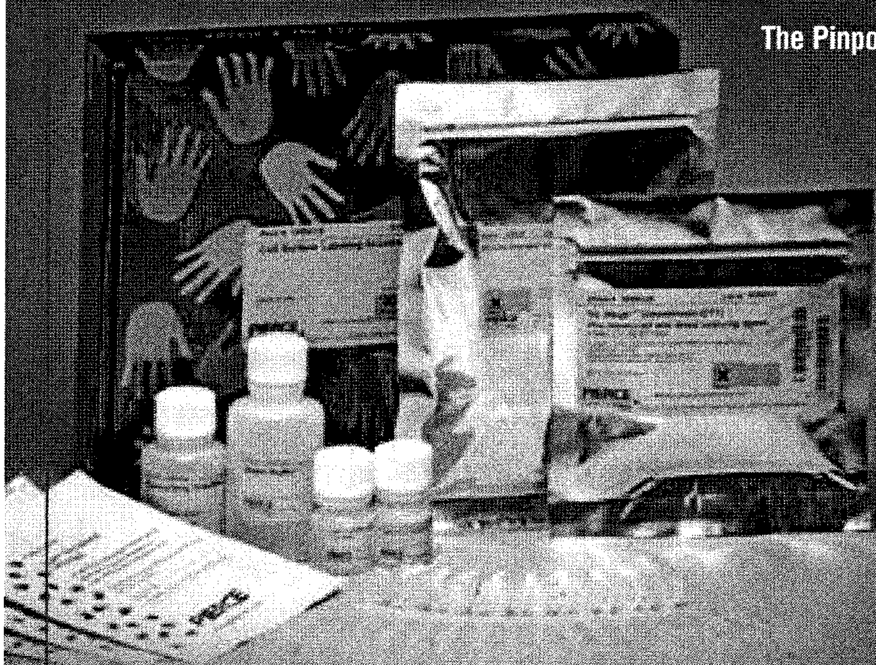
The Pinpoint™ Cell Surface Protein Isolation Kit



This complete kit for the convenient biotinylation and purification of mammalian cell surface proteins has become the standard in the industry from the source you trust in protein chemistry, Pierce.

This kit efficiently labels proteins located on the cell surface with a biotin molecule. The cells are gently lysed, and biotin-labeled cell surface proteins are captured in a NeutrAvidin Spin Column. Once the non-labeled proteins are removed, purified cell surface proteins are eluted from the column with DTT.

Highlights:

- **Isolates cell surface proteins** – reduces complexity of total cellular protein
- **Efficiently recovers labeled proteins** – cleavable biotin allows for nearly 100% recovery of isolated cell surface proteins
- **Convenient** – all reagents are provided in the kit with complete instructions
- **Western blot-ready** – proteins recovered in SDS-PAGE buffer are loaded directly onto SDS-polyacrylamide gels
- **Robust system** – protocol designed for diverse cell lines

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PERBIO

Chelators

BioUltra Reagents

Introduction

The Greek origin of the word chelate signifies the plier-like claws of a crab. A chelate is a water-soluble complex between a metal ion and a complexing agent. It usually does not dissociate easily in solution, but forms an inert complex. In labile complexes, however, the metal ion can be readily exchanged. Metal complexes of transition elements are well known here, chelation occurs within a much wider range of elements. Chelating agents yielding soluble metal complexes are also called sequestering agents [1]. A chelating agent has at least two functional groups which donate a pair of electrons to the metal, such as = O, $-NH_2$ or $-COO^-$. Furthermore, these groups must be located so as to allow ring formation with the metal. Chelating agents are widely found in living systems and are of importance in cellular metabolism.

For applications, requiring highest quality products, we offer a range of BioUltra Chelators.

Natural Chelators

- Water
- Carbohydrates, including polysaccharides
- Organic acids with more than one coordination group
- Lipids
- Steroids
- Amino acids and related compounds
- Peptides
- Phosphates
- Nucleotides
- Tetrapyrrols
- Ferrioxamines
- Ionophores, such as gramicidin, monensin, valinomycin
- Phenolics

Synthetic Chelators

- 2,2'-Bipyridyl
- Dimercaptopropanol
- Ethylenediaminotetraacetic acid, EDTA Ethylenedioxy-diethylene-dinitrilo-tetraacetic acid,
- EGTA, Ethylene glycol-bis-(2-aminoethyl)-N,N,N', N'-tetraacetic acid
- Ionophores Nitrilotriacetic acid, NTA ortho-Phenanthroline
- Salicylic acid
- Triethanolamine, TEA

Chelators as Tools in Biochemistry

Chelating agents have various uses in biochemistry:

1. They constitute nutrient solutions for microorganisms and plants in hydroculture. They may be added to animal food in order to prevent precipitation or absorption of the essential metals [2].
2. They may complex inhibitory heavy metals, either masking them or making their removal possible, e.g. in buffer solutions or in the preparation of dialysis tubings.
3. Chelators may reduce interference of heavy metals when specific metal indicators are used for the determination of intracellular cation concentration, e.g. the measurement of Ca^{2+} with QUIN or FURA [3].
4. Some chelators are important reagents in the quantitative complexometric determination of metals [4].
5. In chelation affinity chromatography, elution is carried out with EDTA or EGTA for a quick group-specific elution [7]

	EDTA	EGTA	HEDTA	NTA
Ag(I)	7.32	6.88	6.71	5.16
Ca(II)	10.96	11.00	8.14	6.41
Cd(II)	16.46	16.70	13.6	9.54
Co(II)	16.31	12.50	14.4	10.38
Cr(III)	23.40			>10
Cu(II)	18.80	17.88	17.55	12.96
Fe(II)	14.33	11.92	12.2	8.84
Fe(III)	25.1	20.5	19.8	15.87
Hg(I)	21.8	23.12	20.1	14.6
Li(I)	2.79	1.17		2.51
Mg(II)	8.69	5.21	7.0	5.46
Mn(II)	14.04	12.3	10.7	7.44
Na(I)	1.66	1.38		2.15

Ni(II)	18.62	13.55	17.0	11.54
Pb(II)	18.04	14.71	15.5	11.39
Sn(II)	18.3	23.85		
Tl(III)	22.5			18
Zn(II)	16.50	14.5	14.5	10.67

Physicochemical Data of Some Complexanes

The term complexane has been recommended by IUPAC for EDTA and other aminopolycarboxylic acids of related structure. The pK values of some complexanes are given below.
In the table above, the absolute stability constants of various metal complexes of these complexanes are reported. As mentioned above these data permit calculation the apparent stability constants of these complexes at any pH. Data were taken from reference [6].

	EDTA	EGTA	HEDTA	NTA
pk_1	1.99	2.00	2.51	1.89
pk_2	2.67	2.65	5.31	2.49
pK_3	6.16	8.85	9.86	9.73
pK_4	10.26	9.46		

EDTA Ethylenediamine-tetraacetic acid Disodium salt

EGTA Ethyleneglycol-O, O'-bis(2-aminoethyl)-N, N, N', N'-tetraacetic acid

HEDTA N-(2-Hydroxyethyl)ethylenediamine-N, N', N'-triacetic acid Trisodium salt

NTA Nitrilotriacetic acid

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Chelators

Reset Table

Product #	Product Name	Purity	Grade
251380	L-(+)-Tartaric acid ACS reagent, ≥99.5%	99.5	ACS reagent
247006	5-Sulfosalicylic acid dihydrate ACS reagent, ≥99%	99, 101	ACS reagent
247561	Ammonium hydrogencitrate 98%, ACS reagent	98, 103	ACS reagent
221716	Ammonium oxalate monohydrate ACS reagent, ≥99%	99, 101	ACS reagent
251275	Citric acid ACS reagent, ≥99.5%	99, 99.5, 102	ACS reagent
E9884	Ethylenediaminetetraacetic acid ACS reagent, 99.4-100.06%, powder	99.4, 100.06	ACS reagent
E4884	Ethylenediaminetetraacetic acid disodium salt dihydrate ACS reagent, 99.0-101.0%	99, 101	ACS reagent
223425	Potassium oxalate monohydrate ACS reagent, 99%	98.5, 99, 101	ACS reagent
217255	Potassium sodium tartrate tetrahydrate ACS reagent, 99%	99, 102	ACS reagent
S4641	Sodium citrate tribasic dihydrate ACS reagent, ≥99.0%	99	ACS reagent
228729	Sodium L-tartrate dibasic dihydrate ≥99%, ACS reagent	99, 101	ACS reagent
223433	Sodium oxalate ACS reagent, ≥99.5%	99.5	ACS reagent
03680	Ethylenediaminetetraacetic acid disodium salt dihydrate puriss. p.a., for complexometry, ACS reagent, ≥99.0% (KT)	99	ACS reagent, Analytical, Complexometry, Puriss, Puriss P.A.
95309	L-(+)-Tartaric acid puriss. p.a., ACS reagent, ≥99.5%	99.5	ACS reagent, Analytical, Puriss, Puriss P.A.
09831	Ammonium citrate dibasic puriss. p.a., ACS reagent, ≥99.0% (T)	99	ACS reagent, Analytical, Puriss, Puriss P.A.
09900	Ammonium oxalate monohydrate puriss. p.a., ACS reagent, ≥99.0% (RT)	99	ACS reagent, Analytical, Puriss, Puriss P.A.
27488	Citric acid puriss. p.a., ACS reagent, anhydrous, ≥99.5% (T)	99.5	ACS reagent, Analytical, Puriss, Puriss P.A.
60426	Potassium oxalate monohydrate puriss. p.a., ACS reagent, ≥99.5% (RT)	99.5	ACS reagent, Analytical, Puriss, Puriss P.A.
60412	Potassium sodium tartrate tetrahydrate puriss. p.a., ACS reagent, ≥99.0% (NT)	99	ACS reagent, Analytical, Puriss, Puriss P.A.
71405	Sodium citrate tribasic dihydrate puriss. p.a., ACS reagent, ≥99.0% (NT)	99	ACS reagent, Analytical, Puriss, Puriss P.A.
71800	Sodium oxalate puriss. p.a., ACS reagent, ≥99.5% (RT)	99.5	ACS reagent, Analytical, Puriss, Puriss P.A.
71401	Sodium citrate tribasic dihydrate *BioUltra*, for luminescence, ≥99.0% (NT)	99	Analytical
03779	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid puriss. p.a., for complexometry, ≥99.0% (T)	99	Analytical, Complexometry, Puriss, Puriss P.A.
03610	Ethylenediaminetetraacetic acid puriss. p.a., for complexometry, ≥99.0% (KT)	99	Analytical, Complexometry, Puriss, Puriss P.A.
61333	Sodium citrate tribasic dihydrate puriss. p.a., eluent additive for LC-MS		Analytical, Liquid Chromatography (LC), Mass Spectrometry (MS), Puriss P.A.
63067	Magnesium citrate tribasic nonahydrate purum p.a., for microbiology, ≥95.0% (KT)	95	Analytical, Microbiology, Purum, Purum P.A.
95310	L-(+)-Tartaric acid puriss., ≥99.5% (T)	99.5	Analytical, Puriss
25517	Sodium tartrate dibasic dihydrate puriss., meets analytical specification of E335, ≥99%	99	Analytical, Puriss
03640	Ethylenediaminetetraacetic acid diammonium salt puriss. p.a., ≥99.0% (KT)	99	Analytical, Puriss, Puriss P.A.
03660	Ethylenediaminetetraacetic acid dipotassium salt dihydrate puriss. p.a., ≥99.0% (KT)	99	Analytical, Puriss, Puriss P.A.
60592	Potassium tetraoxalate dihydrate puriss. p.a., ≥99.5% (RT)	99.5	Analytical, Puriss, Puriss P.A.
71993	Sodium tartrate dibasic dihydrate puriss. p.a., ≥99.0% (NT)	99	Analytical, Puriss, Puriss P.A.
03780	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid purum, ≥98.0% (T)	98	Analytical, Purum
03620	Ethylenediaminetetraacetic acid purum, ≥98.0% (KT)	98	Analytical, Purum
03662	Ethylenediaminetetraacetic acid dipotassium salt dihydrate purum, ≥97.0% (KT)	97	Analytical, Purum
03685	Ethylenediaminetetraacetic acid disodium salt dihydrate purum, ≥97.0% (KT)	97	Analytical, Purum
03665	Ethylenediaminetetraacetic acid tripotassium salt dihydrate purum, ≥96.0% (KT)	96	Analytical, Purum

03710	Ethylenediaminetetraacetic acid trisodium salt dihydrate purum, ≥98.0% (KT)	98	Analytical, Purum
60594	Potassium tetraoxalate dihydrate purum, ≥97.0% (RT)	97	Analytical, Purum
83181	Pyromellitic acid purum, ≥97.0% (T), for fluorescence	97	Analytical, Purum
09834	Ammonium citrate dibasic purum p.a., ≥98.0% (T)	98	Analytical, Purum, Purum P.A.
09901	Ammonium oxalate monohydrate purum p.a., ≥98.0% (RT)	98	Analytical, Purum, Purum P.A.
09984	Ammonium tartrate dibasic purum p.a., crystallized, ≥99.0% (T)	99	Analytical, Purum, Purum P.A.
62485	Lithium citrate tribasic tetrahydrate purum p.a., ≥99.0% (NT)	99	Analytical, Purum, Purum P.A.
60215	Potassium citrate monobasic purum p.a., ≥98.0% (NT)	98	Analytical, Purum, Purum P.A.
60367	Potassium D-tartrate monobasic purum p.a., ≥99.0%	99	Analytical, Purum, Purum P.A.
60425	Potassium oxalate monohydrate purum p.a., ≥99.0% (RT)	99	Analytical, Purum, Purum P.A.
60410	Potassium sodium tartrate tetrahydrate purum p.a., crystallized, ≥99.0% (NT)	99	Analytical, Purum, Purum P.A.
71680	Sodium bitartrate monohydrate purum p.a., ≥98.0% (T)	98	Analytical, Purum, Purum P.A.
71498	Sodium citrate monobasic purum p.a., anhydrous, ≥99.0% (T)	99	Analytical, Purum, Purum P.A.
71406	Sodium citrate tribasic dihydrate purum p.a., ≥99.0% (NT)	99	Analytical, Purum, Purum P.A.
71801	Sodium oxalate purum p.a., ≥99.0% (RT)	99	Analytical, Purum, Purum P.A.
71995	Sodium tartrate dibasic dihydrate purum p.a., ≥98.0% (NT)	98	Analytical, Purum, Purum P.A.
E6758	Ethylenediaminetetraacetic acid anhydrous, crystalline, cell culture tested	98.5	Anhydrous, Cell Culture
EDS	Ethylenediaminetetraacetic acid anhydrous, ~99% (titration)	99	Anhydrous, Sigma
27485	Citric acid *BioUltra*, for luminescence, anhydrous, ≥99.5% (T)	99.5	BioChemika
95308	L-(+)-Tartaric acid *BioUltra*, ≥99.5% (T)	99.5	BioChemika, BioUltra
86193	5-Sulfosalicylic acid dihydrate *BioUltra*, ≥99.0% (T)	99	BioChemika, BioUltra
09833	Ammonium citrate dibasic *BioUltra*, ≥99.0% (T)	99	BioChemika, BioUltra
09898	Ammonium oxalate monohydrate *BioUltra*, ≥99.5% (RT)	99.5	BioChemika, BioUltra
09985	Ammonium tartrate dibasic *BioUltra*, ≥99.5% (T)	99.5	BioChemika, BioUltra
70631	Ammonium tartrate dibasic solution *BioUltra*, 2 M in H_2O (colorless solution at 20 °C)		BioChemika, BioUltra
27487	Citric acid *BioUltra*, anhydrous, ≥99.5% (T)	99.5	BioChemika, BioUltra
03778	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid *BioUltra*, ≥99.0% (T)	99	BioChemika, BioUltra
03609	Ethylenediaminetetraacetic acid *BioUltra*, ≥99.0% (KT)	99	BioChemika, BioUltra
03639	Ethylenediaminetetraacetic acid diammonium salt *BioUltra*, ≥99.5% (KT)	99.5	BioChemika, BioUltra
03659	Ethylenediaminetetraacetic acid dipotassium salt dihydrate *BioUltra*, ≥99.0% (KT)	99	BioChemika, BioUltra
03679	Ethylenediaminetetraacetic acid disodium salt dihydrate *BioUltra*, ≥99.0% (KT)	99	BioChemika, BioUltra
03699	Ethylenediaminetetraacetic acid tetrasodium salt hydrate *BioUltra*, ≥99.0% (KT)	99	BioChemika, BioUltra
03664	Ethylenediaminetetraacetic acid tripotassium salt dihydrate *BioUltra*, ≥99.0% (KT)	99	BioChemika, BioUltra
03709	Ethylenediaminetetraacetic acid trisodium salt trihydrate *BioUltra*, ≥99.0% (KT)	99	BioChemika, BioUltra
62484	Lithium citrate tribasic tetrahydrate *BioUltra*, ≥99.5% (NT)	99.5	BioChemika, BioUltra
63066	Magnesium citrate tribasic nonahydrate *BioUltra*, ≥98.0% (calc. based on dry substance, KT)	98	BioChemika, BioUltra
40105	N,N-Dimethyldecylamine N-oxide solution *BioUltra*, 0.1 M in H_2O		BioChemika, BioUltra
40231	*N,N*-Dimethyldodecylamine *N*-oxide solution *BioUltra*, ~0.1 M in H_2O		BioChemika, BioUltra
72559	Nitrilotriacetic acid *BioUltra*, ≥99.0% (T)	99	BioChemika, BioUltra
60214	Potassium citrate monobasic *BioUltra*, ≥99.0% (dried material, NT)	99	BioChemika, BioUltra
89306	Potassium citrate tribasic solution *BioUltra*, 1 M in H_2O		BioChemika, BioUltra
60366	Potassium D-tartrate monobasic *BioUltra*, ≥99.0% (T)	99	BioChemika, BioUltra
60424	Potassium oxalate monohydrate *BioUltra*, ≥99.0% (RT)	99	BioChemika, BioUltra
60487	Potassium peroxodisulfate *BioUltra*, ≥99.0% (RT)	99	BioChemika, BioUltra
81028	Potassium sodium tartrate solution *BioUltra*, 1.5 M in H_2O		BioChemika, BioUltra
60413	Potassium sodium tartrate tetrahydrate *BioUltra*, ≥99.5% (NT)	99.5	BioChemika, BioUltra
60589	Potassium tetraoxalate dihydrate *BioUltra*, ≥99.5% (RT)	99.5	BioChemika, BioUltra
83179	Pyromellitic acid hydrate *BioUltra*, ≥99.0% (calc. based on dry substance, T), ~2 mol/mol water	99	BioChemika, BioUltra
71679	Sodium bitartrate monohydrate *BioUltra*, ≥99.0% (T)	99	BioChemika, BioUltra
71497	Sodium citrate monobasic *BioUltra*, anhydrous, ≥99.5% (T)	99.5	BioChemika, BioUltra
71404	Sodium citrate tribasic dihydrate *BioUltra*, ≥99.0% (NT)	99	BioChemika, BioUltra

71799	Sodium oxalate *BioUltra*, ≥99.5% (RT)	99.5	BioChemika, BioUltra
71994	Sodium tartrate dibasic dihydrate *BioUltra*, ≥99.0% (NT)	99	BioChemika, BioUltra
79299	Sodium tartrate dibasic solution *BioUltra*, 1.5 M in H_2O (colorless solution at 20 ° C)		BioChemika, BioUltra
83273	Citrate Concentrated Solution *BioUltra*, for molecular biology, 1 M in H_2O		BioChemika, BioUltra, Molecular Biology
03777	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid *BioUltra*, for molecular biology, ≥99.0% (T)	99	BioChemika, BioUltra, Molecular Biology
03677	Ethylenediaminetetraacetic acid disodium salt dihydrate *BioUltra*, for molecular biology, ≥99.0% (T)	99	BioChemika, BioUltra, Molecular Biology
03690	Ethylenediaminetetraacetic acid solution *BioUltra*, for molecular biology, pH 8.0, ~0.5 M in H_2O		BioChemika, BioUltra, Molecular Biology
71402	Sodium citrate tribasic dihydrate *BioUltra*, for molecular biology, ≥99.5% (NT)	99.5	BioChemika, BioUltra, Molecular Biology
C2404	Citric acid cell culture tested, anhydrous		Cell Culture
E8008	Ethylenediaminetetraacetic acid solution 0.02% in DPBS (0.5 mM), cell culture tested, sterile-filtered		Cell Culture
C4540	Citric acid plant cell culture tested		Cell Culture, Plant
E6635	Ethylenediaminetetraacetic acid disodium salt dihydrate Sigma Grade, plant cell culture tested, ~99%	99	Cell Culture, Plant, Sigma
S3147	5-Sulfosalicylic acid dihydrate for electrophoresis, ≥99%	99	Electrophoresis
86192	5-Sulfosalicylic acid dihydrate *BioChemika*, for electrophoresis, ≥99.0% (T)	99	Electrophoresis
E5513	Ethylenediaminetetraacetic acid disodium salt dihydrate for electrophoresis, ≥99%	99	Electrophoresis
03682	Ethylenediaminetetraacetic acid disodium salt dihydrate puriss. p.a., for HPLC, ≥99.0% (KT)	99	HPLC
E3889	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid for molecular biology, ≥97.0%	97	Molecular Biology
E5134	Ethylenediaminetetraacetic acid disodium salt dihydrate for molecular biology, ≥99%	99	Molecular Biology
E7889	Ethylenediaminetetraacetic acid disodium salt solution for molecular biology, 0.5 M		Molecular Biology
C8532	Sodium citrate tribasic dihydrate Molecular Biology, ~99% (capillary GC)	99	Molecular Biology
95318	L-(+)-Tartaric acid Ph Eur		Ph. Eur., USP/NF
71409	Sodium citrate tribasic dihydrate Ph Eur		Ph. Eur., USP/NF
C0909	Sodium citrate tribasic dihydrate suitable for amino acid analysis, ≥98% (titration), powder	98	Proteomics
ED	Ethylenediaminetetraacetic acid purified grade, ~99%, powder	99	Purified
C0759	Citric acid reagent grade		Reagent
ED2SS	Ethylenediaminetetraacetic acid disodium salt dihydrate reagent grade, ~99% (titration)	99	Reagent
N9877	Nitrilotriacetic acid Sigma Grade, ≥99%	99	Sigma
T1807	L-(+)-Tartaric acid SigmaUltra	98	SigmaUltra
S7422	5-Sulfosalicylic acid dihydrate SigmaUltra, ≥99.0%	99	SigmaUltra
A2956	Ammonium tartrate dibasic SigmaUltra		SigmaUltra
E0396	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid SigmaUltra, ≥97%	97	SigmaUltra
E1644	Ethylenediaminetetraacetic acid disodium salt dihydrate SigmaUltra, ~99%	99	SigmaUltra
P0963	Potassium oxalate monohydrate SigmaUltra, ≥98.5%	98.5	SigmaUltra
S6170	Potassium sodium tartrate tetrahydrate SigmaUltra, ≥99%	99	SigmaUltra
C3434	Sodium citrate tribasic dihydrate SigmaUltra, ≥99.0% (titration)	99	SigmaUltra
O0136	Sodium oxalate SigmaUltra		SigmaUltra
S4797	Sodium tartrate dibasic dihydrate SigmaUltra, ≥99.0%	99	SigmaUltra
P2347	Potassium sodium tartrate tetrahydrate meets USP testing specifications		USP
C1857	Citric acid meets USP testing specifications, anhydrous	99.5, 100.5	USP, USP/NF
E0399	Edetate disodium meets USP testing specifications		USP, USP/NF
S1804	Sodium citrate meets USP testing specifications		USP, USP/NF
S2130	5-Sulfosalicylic acid dihydrate *ReagentPlus*®, ≥99%	99	
C1883	Ammonium citrate dibasic ~98% (capillary GC)	98	
A4767	Ammonium tartrate dibasic crystalline		
C83155	Citric acid 99%	99	
E4378	Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid ≥97.0%	97	

ED2P	Ethylenediaminetetraacetic acid dipotassium salt dihydrate ≥98%	98
E0270	Ethylenediaminetetraacetic acid tripotassium salt dihydrate 98% (titration)	98
T2032	Potassium D-tartrate monobasic	
O0501	Potassium oxalate monohydrate ≥98%	98
S2377	Potassium sodium tartrate tetrahydrate *ReagentPlus*®, ≥99%	99
C7254	Sodium citrate tribasic dihydrate ≥98%	98
O0626	Sodium oxalate ≥99% (titration)	99
T6521	Sodium tartrate dibasic dihydrate 99.0-101.0% (non-aqueous titration)	99, 101

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Protein Labeling

Introduction to Protein Labeling

Proteins can be covalently modified in many ways to suit the purpose of a particular assay. A wide variety of immunological and other protein methodologies involve the use of labeled antibodies or other proteins. Enzymes, biotin, fluorophores and radioactive isotopes are commonly used in biotechnology applications to provide a detection signal. They can be conjugated to antibodies, avidin, streptavidin, Fc binding proteins such as Protein A or G, or other proteins. The labeled molecule can then be used in a variety of detection systems.

Most protein labeling methods involve one of four common target strategies. The most common target for protein labeling is primary amines, which are found primarily on lysine residues. They are abundant, widely distributed and easily modified because of their reactivity and their location on the surface of proteins. The second most common target is sulfhydryls, which exist in proteins in reducing conditions but more often are present in oxidized form as disulfide bonds. Because sulfhydryls are less abundant than primary aminies, targeting them results in more specific conjugates. If sulfhydryls are not available for labeling, they may be introduced into a protein by reduction of disulfides, chemical modification of primary amines or point mutation to introduce cysteine residues. Two other common targets are carboxyls and carbohydrates. Carboxyls, like primary amines, are abundant and easily accessible. However, they do not react as readily as amines and coupling to them requires the cross-linker EDC (EDAC). Carbohydrate moieties are present on glycoproteins. If the glycosylation sites are not integral to a given protein function, they can often be modified without significantly altering protein activity. Labeling carbohydrates is a twostep process because the carbohydrates must first be oxidized to create reactive aldehydes.

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Biotinylation

The highly specific interaction of avidin with the small vitamin biotin can be a useful tool in assay systems designed to detect and target biological analytes. The extraordinary affinity of avidin for biotin allows biotin-containing molecules in a complex mixture to be discretely bound with avidin conjugates.

Avidin is a glycoprotein found in the egg white and tissues of birds, reptiles and amphibia. This protein contains four identical subunits having a combined mass of 67,000-68,000 daltons. Each subunit consists of 128 amino acids and binds one molecule of biotin. Avidin is highly glycosylated: carbohydrate accounts for about 10% of the total mass of avidin. Avidin has a basic isoelectric point (pI) of 10-10.5 and is very soluble in water and aqueous salt solutions. Avidin is stable over a wide range of pH and temperature. Extensive chemical modification has little effect on the activity of avidin, making it useful for detection and protein purification.

Streptavidin is another biotin-binding protein that is isolated from *Streptomyces avidinii* and has a mass of 60,000 daltons. In contrast to avidin, streptavidin has no carbohydrate and has an acidic isoelectric point (pI = 5). Pierce products use a recombinant form of streptavidin with a mass of 53,000 daltons and a near-neutral pI. Streptavidin is much less soluble in water than avidin and can be crystallized from water or 50% isopropyl alcohol. There are considerable differences in the composition of avidin and streptavidin, but they are remarkably similar in other respects. Streptavidin is also a tetrameric protein, with each subunit binding one molecule of biotin with a similar affinity to that of avidin. Guanidinium chloride will dissociate avidin and streptavidin into subunits, but streptavidin is more resistant to dissociation.

NeutrAvidin has a mass of 60,000 daltons and is a deglycosylated form of avidin. As a result, lectin binding is reduced to undetectable levels, yet biotin-binding affinity is retained because the carbohydrate is not necessary for this activity. NeutrAvidin Protein offers the advantages of a neutral pI to minimize nonspecific adsorption, along with lysine residues that remain available for derivatization or conjugation. NeutrAvidin Protein yields the lowest nonspecific binding among the known biotin binding proteins.

Biotin, a 244 dalton vitamin found in tissue and blood, binds with high affinity to avidin, streptavidin and NeutrAvidin protein. Since biotin is a relatively small molecule, it can be conjugated to many proteins without significantly altering the biological activity of the protein. A protein can be reacted with several molecules of biotin that, in turn, can each bind a molecule of avidin. This greatly increases the sensitivity of many assay procedures. The avidin-biotin interaction is the strongest known noncovalent biological interaction ($K_a = 10^{15}\ M^{-1}$) between protein and ligand. The bond formation between biotin and avidin is very rapid and, once formed, is unaffected by most extremes of pH, organic solvents and other denaturing agents. The avidin-biotin complex can withstand up to 3 M guanidine•HCl. Biotin can be released by 8 M guanidine•HCl at pH 1.5 or by autoclaving. Avidin can be considered to have essentially non-reversible biotin-binding properties because bound biotin can be released only by denaturing the subunits of the proteins.

Pierce has developed Monomeric Avidin, which also binds specifically to biotin, to allow the purification of fully functional biotinylated proteins. Unlike other biotin-binding proteins that require harsh, denaturing conditions to elute and recover bound molecules, Monomeric Avidin binds reversibly to biotin and allows gentle elution and recovery of biotinylated molecules using a solution of 2 mM free biotin. This makes it possible to harness the avidin-biotin interaction as a purification tool to recover functional proteins and other biological molecules.

Biotin can be easily attached to most proteins and other molecules. Biotinylation reagents are available for targeting a variety of specific functional groups, including primary amines, sulfhydryls, carboxyls and carbohydrates. Photoreactive biotin compounds that react nonspecifically upon photoactivation are also available and expand the scope of molecules that may be labeled with biotin. The variety of biotinylation reagents with different functional group specificities is extremely useful, allowing one to choose a reagent that does not inactivate the target macromolecule. Several cleavable or reversible biotinylation reagents are also available and allow specific elution of the biotinylated molecule from biotin-binding proteins. Cleavable/reversible biotinylation strategies

substantially expand the range of applications for avidin-biotin chemistry.

The most frequently-used biotinylation reagents, *N*-hydroxysuccinimide (NHS) esters and *N*-hydroxysulfosuccinimide (sulfo-NHS) esters, react with primary amines. The functional groups available on the surface of the protein to be biotinylated may not be known. However, with most proteins, it is safe to assume that primary amines are available and accessible for biotinylation. The likelihood that primary amines are available for coupling increases as molecular weight increases. For example, BSA contains 59 primary amines; 30-35 of these are present on the surface and can be reacted with NHS-esters.

While NHS-esters of biotin are most frequently used, they are not necessarily the best for a particular application. If the primary amines on a protein are not fully reacted, reaction with NHS-esters of biotin will result in a random distribution of biotin on the surface of the protein. If a particular primary amine is critical to the biological activity of the protein, modification of this critical amine may result in the loss of its biological activity. Depending on the extent of biotinylation, complete loss of activity may occur when coupling to amines.

When the protein of interest is an antibody, it is advantageous to biotinylate in a manner that will maintain immunological activity. If the antibody contains a lysine rich antigen binding site, amine-reactive agents may inhibit antigen-binding. One solution is to use biotin derivatives that react with sulfhydryl groups. By reducing the immunoglobulin under mild conditions using 2-Mercaptoethylamine (Product # 20408), biotinylation can be targeted to free sulfhydryls generated from the hinge region. Another solution is to use a biotin derivative that reacts with aldehydes. Aldehydes can be generated on antibodies and other glycoproteins by oxidation of carbohydrates with periodate.

It is often important to determine the extent of biotin modification after a biotinylation reaction is complete. Measuring biotin incorporation into macromolecules can aid in optimizing a particular avidin-biotin assay system, and it also can be used to ensure reproducibility in the biotinylation process. The most common method of measuring the degree of biotinylation makes use of the 4´-hydroxyazobenzene-2-carboxylic acid (HABA) dye assay. In the absence of biotin, the dye is capable of forming specific noncovalent complexes with avidin at its biotin-binding sites. Upon binding to avidin in aqueous solution, HABA exhibits a characteristic absorption band at 500 nm (ε = 35,500 $M^{-1}cm^{-1}$, expressed as per mole of HABA bound). The addition of biotin to this complex results in displacement of HABA from the binding site since the association constant of the avidinbiotin interaction (1.3×10^{15} M^{-1}) is much greater than that for avidin-HABA (6×10^{6} M^{-1}). As HABA is displaced, the absorbance of the complex decreases proportionately. Thus, the amount of biotin present in the solution can be determined by plotting the avidin-HABA absorbance at 500 nm versus the absorbance with increasing concentrations of added biotin. Comparing an unknown biotincontaining sample to this standard response curve allows determination of the biotin concentration in the sample. The HABA-avidin system is available in an easy-to-use format in the EZ Biotin Quantitation Kit (Product # 28005).

Amine Specific Biotinylation Reagents
The most common target for modifying protein molecules is the amine group, which is present on the vast majority of proteins because of the abundance of lysine side chain ε-amines and N-terminal α-amines. Based on water solubility, amine-reactive biotinylation reagents can be divided into two groups: NHS esters and sulfo-NHS esters. For reactions in aqueous solution, NHS esters must first be dissolved in an organic solvent, then diluted into the aqueous reaction mixture. The most commonly used organic solvents for this purpose are DMSO and DMF. These solvents are compatible with most proteins at 20% final concentration. Sulfo-NHS esters of biotin are soluble up to approximately 10 mM in water. Sulfo-NHS esters should be dissolved in water just before use, because these compounds are very prone to hydrolysis in solution. Even with the NHS esters, the solvents used to initially dissolve them are hygroscopic and promote hydrolysis of the NHS ester. The reaction chemistries of NHS and sulfo-NHS esters are essentially identical. As demonstrated in Figure 1, an amide bond is formed, and NHS or sulfo-NHS are leaving groups in the reaction.

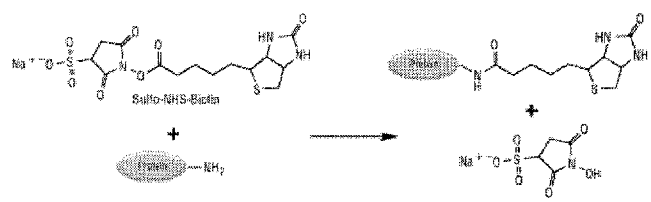

Figure 1. The reaction of Sulfo-NHS Biotin (water-soluble) with a primary amine.

Because the target for the ester is the deprotonated form of the primary amine, the reaction becomes significant at or above neutral pH values when the amine is able to react with the ester by nucleophilic attack. Hydrolysis of the NHS ester is a major competing reaction in aqueous solution, and the rate of hydrolysis increases with increasing pH. NHS- and sulfo-NHS esters have a half-life of hydrolysis of 2-4 hours at pH 7. This half-life decreases to just a few minutes at pH 9.

There is considerable variation in the actual conditions used for conjugating NHS esters (or sulfo-NHS esters) to primary amines. Commonly used incubation temperatures range from 4-37°C; reaction mixture pH values range from 7-9; and incubation times range from a few minutes to overnight. Buffers containing primary amines (such as Tris or glycine) must be avoided because they compete with the reaction. Preparing an ideal conjugate is largely dependent on the degree of biotin incorporation, and a particular set of conditions will result in a conjugate with optimum properties for a specific application. Because of the variability among proteins, especially in the number of amines available for conjugation, reaction conditions that are optimal for one protein may not be optimal for another protein.

Sulfo-NHS-Biotin (Product # 21217), Sulfo-NHS-LC-Biotin (Product # 21335), Sulfo-NHS-LC-LCBiotin (Product # 21338) and Sulfo-NHS-SS-Biotin (Product # 21331) are water-soluble NHS esters of biotin. Their water solubility results from the presence of the sulfonate ($-SO_3$ -) group on the *N*-hydroxysuccinimide ring and eliminates the need to dissolve the reagent in an organic solvent before use. These compounds are used for their convenience and for applications that cannot tolerate organic solvents. Sulfo-NHS esters of biotin are also recommended for use as cell surface biotinylation reagents because the charged sulfonate group limits penetration of the plasma membrane, thus restricting biotinylation to the cell surface. Sulfo-NHS-LC-Biotin has a long chain spacer arm of 22.4 Å which reduces steric hindrance associated with binding multiple biotinylated molecules on one avidin and results in enhanced detection sensitivity. Sulfo-NHS-LC-Biotin usually is a first-choice biotinylation reagent because of its long chain spacer arm and its reactivity toward primary amines. Sulfo-NHS-LC-LC-Biotin may be used when steric hindrance is a major concern and the longest possible spacer arm is required. Sulfo-NHS-SS-Biotin is a thiol-cleavable water-soluble reagent. Sulfo-NHS-SS-Biotin is used to reversibly biotinylate a protein or peptide. First, the macromolecule is reacted with Sulfo-NHS-SS-Biotin. The biotinylated macromolecule is then used in a detection or purification system. The biotinylated macromolecule interacts with avidin in the normal manner; however, under reducing conditions (50 mM dithiothreitol, 100 mM 2-mercaptoethanol or 1% sodium borohydride) the biotin moiety can be cleaved from the macromolecule releasing the molecule into solution.

Pierce also supplies the two most popular biotinylation reagents, Sulfo-NHS-Biotin and Sulfo-NHS-LC-Biotin, as complete protein labeling kits (Product # 21420, 21430). These kits provide all of the required labeling reagents along with an optimized protocol that yields a high labeling efficiency. A complete kit is also available for the biotinylation and purification of cell-surface proteins

(Product # 89881).

NHS-PEO$_4$-Biotin (Product # 21330, 21329) is another water-soluble, amine reactive biotinylation reagent. The solubility results from its spacer arm containing polyethylene oxide (PEO). This reagent has the advantage of conferring increased solubility to the labeled protein molecule. Unlike sulfo-NHS esters, which lose their solubility-enhancing sulfonate group during the labeling reaction, reagents containing a PEO spacer arm transfer their high solubility when coupled to a protein.

NHS-Biotin (Product # 20217), NHS-LC-Biotin (Product # 21336) and NHS-LC-LC-Biotin (Product # 21343) are water-insoluble NHS esters of biotin. These compounds will biotinylate in aqueous solutions if they are first dissolved in an organic solvent (typically DMSO or DMF), then diluted into the aqueous reaction mixture. The solvent acts as a carrier for the biotinylation reagent, forming a microemulsion in the aqueous phase and allowing the biotinylation reaction to proceed. The water insoluble NHS esters of biotin are membrane permeable because they do not possess a charged group. They may be used for biotinylating internal as well as external components of a cell.

NHS-Iminobiotin (Product # 21117) is the guanido analog of NHS-Biotin. NHS-Iminobiotin can be used for applications that require mild dissociation conditions from avidin and for those that cannot tolerate the reducing conditions required to break the disulfide bond of a cleavable biotinylation reagent. At pH 9.5 or greater, avidin binds tightly to iminobiotin; complete dissociation of the complex occurs at pH 4. There are two possible explanations for this binding phenomenon. One reasoning deals with the ionizing character of the cyclic guanido group of 2-iminobiotin. With increasing pH, the affinity for avidin increases. The cyclic guanido group of 2-iminobiotin has a pKa of 11.5 to 12, so avidin may bind iminobiotin only as the deprotonated form. However, the reduced binding affinity at pH values below 6 suggests that an ionizable group on avidin may also be involved. Regardless of the exact mechanism of binding, the pH-dependent avidin binding property of iminobiotin derivatives provides a gentle, reversible option for employing the avidin-biotin system.

PFP-Biotin (Product # 21218) and TFP-PEO-Biotin (Product # 21219) are more reactive than NHS esters and will react with both primary and secondary amines at pH 7-9 to create a stable amide bond. The PEO spacer arm of TFP-PEO-Biotin increases the solubility in aqueous solution of both the reagent and biotinylated molecules.

Sulfhydryl Specific Biotinylation Reagents
Cysteine on the surface of a protein or within a peptide is the most common source of sulfhydryl groups (–SH or thiol groups) in biological molecules. Sulfhydryl group biotinylation often provides an advantage in an application. For example, a protein in a complex mixture can be specifically biotinylated if it is the only one with a free sulfhydryl group on its surface. Also, targeting a sulfhydryl group for biotinylation can be used as a method for preserving the biological activity of a molecule when amines are found at the site of biological activity (eg. antigen binding site of an antibody). Modification of these amines may make these macromolecules inactive, but this complication can be avoided by using derivatives of biotin that react with sulfhydryls. Reactions with these biotinylation reagents should be performed in buffers free of extraneous sulfhydryls. Therefore, substances such as 2-mercaptoethanol, dithiothreitol, glutathione and mercaptoethylamine must be removed before biotinylation.

Proteins, peptides or other molecules to be biotinylated by sulfhydryl-reactive reagents must have a sulfhydryl group available. With peptides, a terminal cysteine is often added during synthesis to target one end of the peptide for modification. However, some cysteine-containing peptides may oxidize in solution and form disulfide bonds, which will not react with sulfhydryl-specific biotinylation reagents. These peptides must be reduced, then separated from excess reducing agent. Alternatively, the primary amine on the N-terminus can be labeled with a protected sulfhydryl group and, immediately after deprotection, the biotinylation reaction performed. If sulfhydryls are not available, they can be generated from disulfides by incubation with a reducing agent. Mercaptoethylamine• HCl (Product # 20408) can be used with IgG or F(ab')$_2$ molecules to cleave the disulfides between the heavy chains, while maintaining both the disulfide linkages between the heavy and light chains and the activity of the antibody. When doing mercaptoethylamine cleavage, EDTA should be included for its antioxidative effect; it chelates trace amounts of metals in solution that promote disulfide bond formation. Sulfhydryl groups generated at the hinge region of IgG molecules are fairly stable in the presence of EDTA. Using nitrogen-purged buffers is an additional precaution used to prevent reoxidation of the sulfhydryls to disulfides. For reducing disulfide bonds between small peptides, Immobilized TCEP Reducing Gel is ideal (Product # 77712). Simply pass the peptide through a prepacked column of immobilized TCEP, and the peptide emerges from the column in reduced form, free from any reducing agent.

Iodoacetyl-LC-Biotin (Product # 21333) and PEO-Iodoacetyl-LC-Biotin (Product # 21334) are sulfhydryl-reactive derivatives of biotin. Iodoacetyl-LC-Biotin is water-insoluble and should be dissolved in a solvent before use in an aqueous reaction mixture. The PEO-Iodoacetyl-LC-Biotin is water-soluble by virtue of its polyethyleneoxide (PEO) spacer arm and may be dissolved directly in aqueous solution. The iodoacetyl group reacts mainly with sulfhydryl groups at pH 7.5-8.5, resulting in a stable thioether bond. Unless precautions are taken, iodoacetyl groups may not be specific for sulfhydryls. The reaction can be directed toward sulfhydryl groups by limiting the molar ratio of iodoacetyl-biotin to protein, such that the concentration of biotin is present at a small excess over the sulfhydryl content. Also, the reaction pH is best maintained in the range of 7.5-8.5. Below pH 9, the reactivity with amine, thioether and imidazole groups is minimized, ensuring modification of only sulfhydryl groups. If there are no cysteines available, the reaction can be directed at imidazoles by adjusting the pH to 6.9-7.0. However, the incubation time must be increased to a week. Histidyl side chains and amino groups react in the unprotonated form and may take part in reactions above pH 5 and pH 7, respectively, although this reaction is much slower than that for sulfhydryls.

Biotin-BMCC (Product # 21900) is a sulfhydryl-reactive, water-insoluble biotinylation reagent with a long spacer arm of 32.6 Å that often enhances the sensitivity of detection. Maleimide-PEO$_2$-Biotin (Product # 21901, 21902) is another sulfhydryl-reactive biotinylation reagent, but because of its PEO spacer arm it is water-soluble. These compounds contain a maleimide functional group, which is more specific for the -SH group than iodoacetyl groups. At pH 7 the maleimide group is 1000 times more reactive toward a sulfhydryl than toward an amine. The reaction of these reagents is carried out at pH 6.5-7.5 since reactivity toward primary amines can occur at higher pH values. Hydrolysis of the maleimide group also increases at higher pH values.

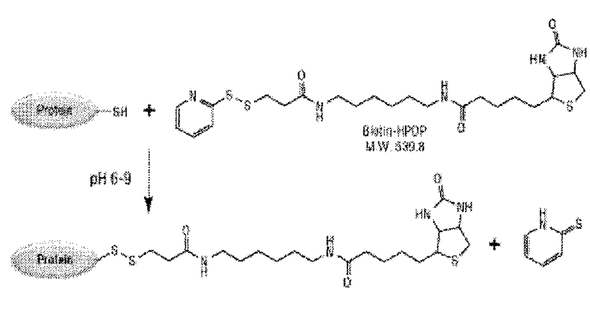

Figure 18. Biotin-HPDP reaction.

Biotin-HPDP (Product # 21341) is a sulfhydryl-reactive, cleavable reagent that offers a key advantage over other cleavable reagents that leave behind a modified side chain (Figure 2). Biotin-HPDP can be completely removed, regenerating the starting protein in its original, unmodified form. The reactive group on this labeling reagent is a pyridyldithiol that reacts with sulfhydryls by disulfide exchange, forming a mixed disulfide linkage. In the process, pyridine-2-thione is released and can be used to monitor the reaction because it absorbs light at 343 nm.

Carbohydrate Reactive Biotinylation Reagents

Oxidative pretreatment of glycoproteins is used to generate reactive aldehydes that react specifically with hydrazide groups. Sialic acid residues on glycoproteins can be specifically oxidized with sodium periodate ($NaIO_4$) using controlled conditions. At 1 mM periodate and a temperature of 0°C, the reaction is restricted primarily to sialic acid residues. Sialic acid residues also can be biotinylated with hydrazide derivatives by pretreatment with neuraminidase to generate galactose groups. The galactose and N-acetylgalactosamine residues on whole cells can be selectively biotinylated with biotin-hydrazides by pretreatment with galactose oxidase. This enzyme will convert the primary hydroxyl groups on these sugars to their corresponding aldehydes.

Mild oxidation of an immunoglobulin with sodium periodate will produce reactive aldehydes from *cis* -diols on the carbohydrate moieties of the Fc portion, which then can be alkylated by these hydrazides. This approach is advantageous for use with antibodies because they become biotinylated in a manner that maintains immunological reactivity. This is an ideal method for biotinylating polyclonal antibodies because they are heavily glycosylated. Monoclonal antibodies may be deficient in glycosylation and success with this method will depend on the extent of glycosylation for a particular antibody.

Temperature, pH of oxidation and the periodate concentration all affect the extent of aldehyde activation and therefore also the extent of labeling with hydrazide derivatives of biotin. Since glycosylation varies with each protein, optimal conditions must be determined for each glycoprotein. Each glycoprotein preparation has an optimum pH for oxidation and an optimum pH for the hydrazide biotinylation. Tris, or other primary amine-containing buffers, are not recommended for use in either the oxidation or biotinylation steps since these buffers react with aldehydes, quenching their reaction with hydrazides.

Biotin Hydrazide (Product # 21339), Biotin-LC-Hydrazide (Product # 21340) and Biocytin Hydrazide (Product # 28020) bind to oxidized carbohydrates through the hydrazide group ($–NH-NH_2$), forming a hydrazone linkage (Figure 3). Biotin Hydrazide and Biotin-LC-Hydrazide are soluble in aqueous buffers up to about 5 mM, and Biocytin Hydrazide is a more soluble derivative. They can also be dissolved first in DMSO (at concentrations up to 50 mM), then diluted into aqueous reaction mixtures. It should be noted that Biotin-Hydrazide and Biotin-LC-Hydrazide are poorly soluble in DMF (less than 5 mM). Hydrazides react spontaneously with oxidized carbohydrates to yield stable hydrazone bonds.

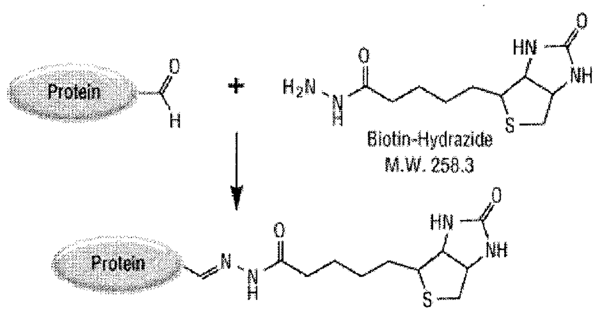

Figure 19. Reaction of biotin hydrazide with an aldehyde.

Carboxyl Reactive Biotinylation Reagents

Biotin PEO-Amine (Product # 21346), Biotin PEO-LC-Amine (Product # 21347) and 5-(Biotinamido) pentylamine (Product # 21345) can each be reacted with carboxyl groups in the form of carboxy termini, aspartate residues or glutamate residues. In addition, any of the hydrazide-derivatized biotin molecules can be reacted with carboxyls using the same conditions. The reaction is mediated by EDC (Product # 22980, 22981), the most common, water-soluble carbodiimide cross-linker. EDC activates carboxyl groups to bind to the $–NH_2$ group from the biotinylation reagent, forming an amide linkage.

Using EDC may result in some polymerization of the peptide or protein if the molecule has both carboxyls and primary amines on its surface. The extent of polymerization can be minimized by decreasing the amount of EDC and/or increasing the amount of the biotin reagent used in the reaction. EDC-mediated reactions are generally performed in an MES buffer at pH 4.5-5 and require just minutes to complete. Buffers containing primary amines (Tris, glycine, etc.) or carboxyls (acetate, citrate, etc.) should be avoided since they will quench the reaction. Phosphate buffers are also not recommended because they reduce the conjugation efficiency, although this effect can be overcome by adding more EDC.

Photoreactive Biotinylation Reagents

Photoactivatable Biotin (Product # 29987) , Psoralen-PEO-Biotin (Product # 29986) and Biotin-LC-ASA (Product # 29982) each contain a photoactivatable group. When exposed to UV light, they become activated and insert nonspecifically into nearby molecules. This allows labeling of proteins, peptides and other molecules that do not contain any of the easily labeled functional groups already discussed. Photoreactive reagents may be used to label proteins and peptides but they are also useful in labeling DNA, RNA and other molecules that do not contain any readily labeled functional groups.

Most photoreactive reagents contain an aryl azide group that is chemi-cally inert until it is exposed to ultraviolet light, causing the formation of a short-lived, reactive aryl nitrene. The half-life of this aryl nitrene inter-mediate is on the order of 10^{-4} seconds. The aryl nitrene reacts rapidly and non-selectively with electron dense sites by addition into double bonds or insertion into active hydrogen bonds. If the aryl-nitrene fails to react, it undergoes ring expansion and becomes reactive toward nucleophiles such as primary amines and sulfhydryls. Photoactivation and insertion into another molecule can be performed in a wide variety of buffer conditions. However, acidic conditions and reducing agents should be avoided since they may lead to inactivation of the aryl azide group.

Psoralen-PEO-Biotin contains a reactive psoralen group and is designed to efficiently label nucleic acids. The psoralen group intercalates into the helix of DNA allowing it to label efficiently and selectively. The psoralen can also stack along with the bases of single-stranded DNA or RNA increasing labeling efficiency and selectivity. Upon photo-activation, psoralen forms a cross-link with the 5,6 double bond of pyrimidine bases and its presence does not interfere with hybridization.

ProFound Label Transfer Sulfo-SBED (Product # 33033) contains two reactive groups, a photoreactive group and an amine reactive NHS-ester, in addition to a biotin moiety. This unique biotinylation reagent finds its greatest use as a label transfer reagent

in the study of protein:protein interactions. For more information on this molecule, visit the Protein Interactions section.

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PRIVACY STATEMENT

PeptideCutter

The cleavage specificities of selected enzymes and chemicals:

A general model of enzymatic cleavage:

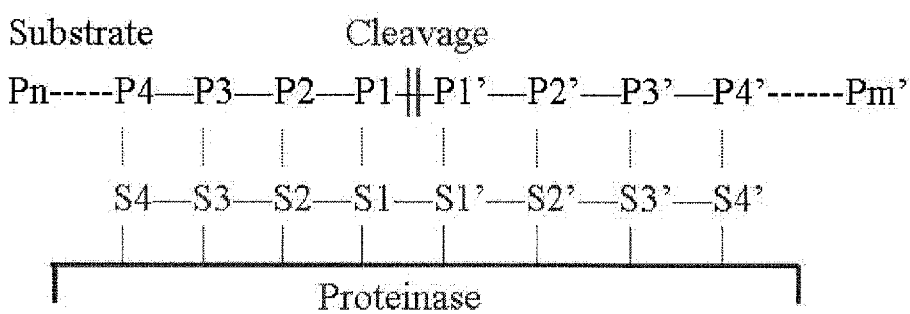

Fig.1 Schematic representation of enzyme-substrate complex with eight binding sites. Positions Pn to Pm' in the substrate are counted from the bond between P1 and P1', where the cleavage occurs.

Subsite nomenclature was adopted from a scheme created by Schechter and Berger (1967, 1968) and used in the following description of enzyme specificities. According to this model, amino acid residues in a substrate undergoing cleavage are designated P1,P2, P3, P4 etc. in the N-terminal direction from the cleaved bond. Likewise, the residues in C-terminal direction are designated P1', P2', P3', P4'. etc. as shown in **Fig.1**. Most of the following rules of protease specificity were adopted from the comprehensive publication of Keil (1992). These rules were verified by checking against information published by Barrett et al. (1998).

Establishing a specificity model for protease cleavage:

A first and obvious approach to obtain information concerning the cleavage specificity of a protease is the characterization of the respective natural substrate. In a next step, standard polypeptides can be used for digestion such as glucagon or insulin chains. However, an ideal polypeptidic substrate should contain all possible 400 combinations of dipeptide bonds, whereas in insulin and glucagon for example only small fractions of these combinations can be found (not to mention the possibilities of tetrapeptide composition when taking into account the sites P2 to P2'). Ideally, the cleavage data would be obtained by digesting all availble proteins in the databases, but this is beyond feasability. A more systematic and complete approach would be to test the proteases with substrates of low molecular weight such as di-, tri, tetrapeptides etc.

Unfortunately, the available data for most proteases is still very incomplete. Only for a few proteases enough information has been accumulated that allow a statistical treatment (for details see Keil (1992) resulting in a more complete and refined picture of cleavage specificity. In the following, specific cleavage preferences of individual enzymes are reported. Only the accordingly derived rules are taken into consideration by the program PeptideCutter. Thus, the user should be aware of the fact, that results obtained by experiments may differ from the predictions made by the PeptideCutter program.

The cleavage specificities of selected enzymes and chemicals:

Arg-C proteinase:

The Arg-C proteinase preferentially cleaves at Arg in position P1. The cleavage behaviour seems to be only moderately affected by residues in position P1' (Keil, 1992).

Asp-N Endopeptidase:

The Asp-N Endopeptidase cleaves specifically bonds with Asp in position P1' (Keil, 1992).

Asp-N Endopeptidase + N-terminal Glu:

The Asp-N Endopeptidase cleaves specifically bonds with Asp or Glu in position P1' (Keil, 1992).

BNPS-Skatole:

BNPS-skatole [2-(2-nitrophenylsulfenyl)-3-methylindole] is a mild oxidant and brominating reagent that leads to polypeptide cleavage on the C-terminal side of tryptophan residues (Information from:http://www.gac.edu/~eb/c5pt1.html).

Caspase 1:

Caspase-1 is acting on Interleukin-1 beta [Precursor] (P01584) to release it by specific cleavage at 116-Asp-|-Ala-117 (YVHDA) and 27-Asp-|-Gly-28 (EADG) bonds. It also hydrolyzes small-molecule substrate such as Ac-Tyr-Val-Ala-Asp-|-NHMec. Generally the substrate/enzyme interaction is located between the positions P4 and P1'. Various different patterns were proposed such as YEVD|X (Talanian et al., 1997) or WEHD|X (Thornberry et al., 1997), where X is any amino acid but Pro, Glu, Asp, Gln, Lys, Arg (Stennicke et al., 2000, Talanian et al., 1997). The pattern implemented for PeptideCutter considers an extended rule based on the study by Earnshaw et al., 1999, to optimise the caspase-1 endoproteolytic specificity, and can be found in the table at the end of this document, describing the possible variations on the different interacting sites from P4 to P'1.

Caspase 2:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1'of which are composed of DVAD|X (Talanian et al.,1997) or DEHD|X (Thornberry et al.,1997), where X is any amino acid but Pro, Glu, Asp, Gln, Lys, Arg (Stennicke et al., 2000, Talanian et al., 1997).

Caspase 3:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1'of which are composed of DMQD|X (Talanian et al.,1997) or DEVD|X (Thornberry et al.,1997), where X is any amino acid but Pro, Glu, Asp, Gln, Lys, Arg (Stennicke et al., 2000, Talanian et al., 1997).

Caspase 4:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1'of which are composed of LEVD|X (Talanian et al.,1997) or (W/L)EHD|X (Thornberry et al.,1997), where X is any amino acid but Pro, Glu, Asp, Gln, Lys, Arg (Stennicke et al., 2000, Talanian et al., 1997).

Caspase 5:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially ooccurs at sites the positions P4 to P1' of which are composed of (W/L)EHD|X (Thornberry et al.,1997).

Caspase 6:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1' of which are composed of VEID|X (Talanian et al.,1997) or VEHD|X (Thornberry et al.,1997), where X is any amino acid but Pro, Glu, Asp, Gln, Lys, Arg (Stennicke et al., 2000, Talanian et al., 1997).

Caspase 7:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially ooccurs at sites the positions P4 to P1' of which are composed of DEVD|X (Talanian et al.,1997;Thornberry et al.,1997), where X is any amino acid but Pro, Glu, Asp, Gln, Lys, Arg (Stennicke et al., 2000, Talanian et al., 1997).

Caspase 8:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1' of which are composed of IETD|X (Talanian et al.,1997) or LETD|X (Thornberry et al.,1997), where X is any amino acid but Pro, Glu, Asp, Gln, Lys, Arg (Stennicke et al., 2000, Talanian et al., 1997).

Caspase 9:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1' of which are composed of LEHD|X (Thornberry et al.,1997).

Caspase 10:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1'of which are composed of IEAD|X (Talanian et al.,1997).

Chymotrypsin:

Chymotrypsin preferentially cleaves at Trp, Tyr and Phe in position P1(high specificity) and to a lesser extent (taken into account when dealing with low specificity chymotrypsin) at Leu, Met and His in position P1 (Keil, 1992). Exceptions to these rules are the following: When Trp is found in position P1, the cleavage is blocked when Met or Pro are found in position P1'at the same time. Furthermore, Pro in position P1' nearly fully blocks the cleavage independent of the amino acids found in position P1. When Met is found in position P1, the cleavage is blocked by the presence of Tyr in position P1'. Finally, when His is located in position P1, the presence of Asp, Met or Trp also blocks the cleavage .

Clostripain (Clostridiopeptidase B):

Clostripain cleaves preferentially at the carboxyl group of arginine residues, i.e. Arg in position P1 (Keil, 1992). This cleavage is not strict, especially when the time of proteolysis is short or when using specific native protein substrates. The cleavage of lysyl-bonds has been reported rarely. Clostripain accepts well substrates containing Lys instead of Arg, however reaction rates are very low in comparison to reactions with Arg containing substrates. This enzymes is reported to be sensitive to the composition of the potential substrate site. However, no rules can be defined. Probably Glu and Asp in position P1' protect against cleavage, as well as the accumulation of positive charge in the positions P1' to P4'.

CNBr:

CNBr cleaves at Met in position P1. (Information from the Cutter of the Prolysis program/Universite de Tours). When CNBr is not applied in large excess, the cleavage may become incomplete. Schroeder et al. (1969) discuss resistance to CNBr that results from Ser or Thr being located in P1' (In this case, the Met residue is converted into a homoserine-residue, thus preventing a cleavage) or P2. In general, this type of cleavage-blocking is prevented by using CNBr in large excess in relation to the number of Met- residues in the sequence.

Enterokinase:

Enterokinase is a serine protease that recognizes the amino acid sequence -Asp-Asp-Asp-Asp-Lys-|-X (Roche) with a high specificity. The enterokinase activates its natural substrate trypsinogen and releases trypsin by cleavage at the C-terminal end of this sequence. The aspartic acid residues can be substituted by glutamic acid. Note that PeptideCutter does not take into account position P5. The implemented motif for Enterokinase is therefore [ND][ND][ND]K-X.

Factor Xa:

(Coagulation factor Xa) Factor Xa is prepared by the activation of its precursor, the mostly inactive Factor X, by the hydrolysis of a specific peptide bond in the amino-terminal region of the heavy chain (Fujikawa et al., 1975). Highly specific for cleavage at Arg in position P1 and Gly in position P2. In general position P3 is occupied by a negatively charged residue (Glu or Asp) and position P4 may be hydrophobic (Ile or Ala). The composition of the P'-sites do not seem to considerably influence the cleavage.

Formic acid:

Cleaves at Asp in position P1 (Li et al., 2001).

Glutamyl endopeptidase:

Mainly three different types are commercially available (Birktoft and Breddam, 1994): GluBI (from *Bacillus licheniformis*, GluSGB (*Streptomyces griseus*), GluV8 (*Streptococcus aureus*, strain *V8*). All of them preferentially cleave at Glu in position P1. When Glu and Asp are found in a directly neighbouring positions the cleavage at Glu in position P1 is preferred a 100-(GluSGP) to around 1000-fold (other two) in relation to Asp in position P1. The nature of the reaction buffer (whether bicarbonate or phosphate) does not seem to have an influence on this ratio, however it has been shown that reactivity in general is enhanced with phosphate in comparison to bicarbonate (Houmard and Drapeau,1972). Generally preferred composition of cleavage site: Asp in position P4, Ala/Val in position P3, Pro/Val in position P2 (GluSGP) or Phe in position P2 (GluBL/GluV8). Disfavouring of Pro in position P3, position P1' and position P2' as well as Asp at position P1'.

Granzyme B:

The present version of the PeptideCutter program considers only the preferred peptide substrate sites as summarized in Earnshaw et al., (1999): Cleavage preferentially occurs at sites the positions P4 to P1'of which are composed of IEPD|X (Thornberry et al.,1997).

Hydroxylamine (NH2OH):

Cleaves at Asn in position P1 and Gly in position P1' (Bornstein & Balian)

Iodosobenzoic acid:

Cleaves at Trp in position P1. (Han et al., 1983)

LysC Lysyl endopeptidase (Achromobacter proteinase I):

Cleaves at Lys in position P1 (Keil, 1992).

LysN Peptidyl-Lys metalloendopeptidase

Cleaves at Lys in position P1' (Keil, 1992).

NTCB +Ni (2-nitro-5-thiocyanobenzoic acid):

Cleaves at Cys in position P1' (Degani and Patchornik, 1974).

Pepsin:

Pepsin preferentially cleaves at Phe, Tyr, Trp and Leu in position P1 or P1'(Keil, 1992). Negative effects on cleavage are excerted by Arg, Lys and His in position P3 and Arg in position P1. Pro has favourable effects when being located in position P4 and position P3, but unfavourable ones when found in positions P2 to P3'. Cleavage is more specific at pH 1.3. Then pepsin preferentially cleaves at Phe and Leu in position P1 with negligible cleavage for all other amino acids in this position. This specificity is lost at pH >= 2.

Proline-endopeptidase:

Proline-endopeptidase preferentially cleaves at Pro in position P1 (Keil, 1992). Proline-endopeptidase may also accept Ala in position P1. With Pro in position P1 the activity is blocked when another Pro is at position P1'. In most cases a basic amino acid (Lys, His, Arg) is found in position P2. It was suggested that this feature is obligatory. Some discrepancies concerning the cleavage specificity were observed in individual cases, however this may be due to impurities or to the fact that Pro-endopeptidases came from different sources and may not be identical.
NOTE: Proline-endopeptidase was reported to cleave only substrates whose sequences do not exceed 30 amino acids. An unusual beta-propeller domain regulates proteolysis: see Fulop et al., 1998.

Proteinase K:

Proteinase K preferentially cleaves at aliphatic of aromatic amino acid residues in position P1 (Keil, 1992). Ala in position P2 enhances the cleavage . The specificity of proteinase K is not always unambiguous.

Staphylococcal peptidase I:

Preferentially cleaves at Glu in position P1, but also, although to a lesser extent, at Asp in position P1 (Keil, 1992). In rare cases Ser can be accepted in position P1. In general, specificity depends strongly on the experimental conditions: the exchange of buffer (not necessarily of pH) can change the cleavage behaviour. When two Glu residues are found in directly neighbouring positions, Staphylococcal peptidase I prefers to cut at Glu in position P1 with another Glu in position P1' instead of the second Glu being located in position P2.

Tobacco etch virus protease:

TEV protease is the common name for the 27 kDa catalytic domain of the Nuclear Inclusion a (NIa) protein encoded by the tobacco etch virus (TEV). Because its sequence specificity is far more stringent than that of factor Xa, thrombin, or enterokinase, TEV protease is a very useful reagent for cleaving fusion proteins. It is also relatively easy to overproduce and purify large quantities of the enzyme. TEV protease recognizes a linear epitope of the general form E-Xaa-Xaa-Y-Xaa-Q-(G/S), with cleavage occurring between Q and G or Q and S. The most commonly used sequence is ENLYFQG (Waugh, 2002, Waugh, TEV protease FAQ)). Note that PeptideCutter does not take into account positions P5 and P6. The implemented motif for TEV protease is therefore XYXQ-[GS].

Thermolysin:

Thermolysin preferentially cleaves sites with bulky and aromatic residues (Ile, Leu, Val, Ala, Met, Phe) in position P1' (Keil, 1992). Cleavage is favoured with aromatic sites in position P1 but hindered with acidic residues in position P1. Pro blocks when located inposition P2' but not when found in position P1.

Thrombin:

Preferentially cleaves at Arg in position P1 (Keil, 1992).The natural substrate of thrombin is fibrinogen. Optimum cleavage sites are when Arg in position P1 and Gly in position P2 and position P1'. Likewise, when hydrophobic residues are found in position P4 and position P3 , Pro in position P2, Arg in position P1, and non-acidic amino-acids in position P1' and position P2'. A very important residue for its natrual substrate fibrinogen is an Asp in P10 (but this site is neglected in the PeptideCutter program).

Trypsin:

Preferentially cleaves at Arg and Lys in position P1 with higher rates for Arg (Keil, 1992), especially at high pH (but treated equally in the program). Pro usually blocks the action when found in position P1', but not when Lys is in position P1 and Trp is in position P2 at the same time. This blocking of cleavage exerted by Pro in position P1' is also negligible when Arg is in position P1 and Met is in position P2 at the same time (other reports say that the block exhibited by Pro can be circumvented by Glu being in P2).

Furthermore, if Lys is found in position P1 the following situation considerably block the action of trypsin: Either Asp in position P2 and Asp in position P1' or Cys in position P2 and Asp in position P1'or Cys in position P2 and His in position P1' or Cys in position P2 and Tyr in position P1'. A likewise considerable block of trypsin action is seen , when Arg is in P1 and the following situations are found: Either Arg in position P2 and His in position P1' or Cys in position P2 and Lys in position P1' or Arg in position P2 and Arg in positionP1'.

This Arg/Lys specificity is seen very nicely with pure alpha- and beta trypsins. Trypsin preparations with traces of "pseudotrypsin" also cleave considerably at the following amino acids in P1:Phe (except with Glu or Pro in P1'), Tyr (except with Pro and Arg in P1') and Trp (except with Ile, Lys, Pro Val and Trp in P1') Met (with Ala, His, Met, Gln, Ser, Val and Trp in P1') and Cys (with Phe, Gly, Ile, Leu, Val and Trp in P1').

Summary of the cleavage rules:

Cleavage rules

The following enzymes potentially cleave when the respective compositions of the cleavage sites are found. However, there also are some exceptions.

Enzyme name	P4	P3	P2	P1	P1'	P2'
Arg-C proteinase	-	-	-	R	-	-
Asp-N endopeptidase	-	-	-	-	D	-
BNPS-Skatole	-	-	-	W	-	-
Caspase 1	F, W, Y, or L	-	H, A or T	D	not P, E, D, Q, K or R	-
Caspase 2	D	V	A	D	not P, E, D, Q, K or R	-
Caspase 3	D	M	Q	D	not P, E, D, Q, K or R	-
Caspase 4	L	E	V	D	not P, E, D, Q, K or R	-
Caspase 5	L or W	E	H	D	-	-
Caspase 6	V	E	H or I	D	not P, E, D, Q, K or R	-
Caspase 7	D	E	V	D	not P, E, D, Q, K or R	-
Caspase 8	I or L	E	T	D	not P, E, D, Q, K or R	-
Caspase 9	L	E	H	D	-	-
Caspase 10	I	E	A	D	-	-
Chymotrypsin-high specificity (C-term to [FYW], not before P)	-	-	-	F or Y	not P	-
	-	-	-	W	not M or P	-
	-	-	-	F,L or Y	not P	-

Chymotrypsin-low specificity (C-term to [FYWML], not before P)	-	-	-	W	not M or P	-
	-	-	-	M	not P or Y	-
	-	-	-	H	not D,M,P or W	-
Clostripain (Clostridiopeptidase B)	-	-	-	R	-	-
CNBr	-	-	-	M	-	-
Enterokinase	D or N	D or N	D or N	K	-	-
Factor Xa	A,F,G,I,L,T,V or M	D or E	G	R	-	-
Formic acid	-	-	-	D	-	-
Glutamyl endopeptidase	-	-	-	E	-	-
GranzymeB	I	E	P	D	-	-
Hydroxylamine	-	-	-	N	G	-
Iodosobenzoic acid	-	-	-	W	-	-
LysC	-	-	-	K	-	-
NTCB (2-nitro-5-thiocyanobenzoic acid)	-	-	-	-	C	-
Pepsin (pH1.3)	-	not H,K, or R	not P	not R	F,L,W or Y	not P
	-	not H,K, or R	not P	F,L,W or Y	-	not P
Pepsin (pH>2)	-	not H,K or R	not P	not R	F or L	not P
	-	not H,K or R	not P	F or L	-	not P
Proline-endopeptidase	-	-	H,K or R	P	not P	-

Proteinase K	-	-	-	A,E,F,I,L,T,V,W or Y	-	-
Staphylococcal peptidase I	-	-	not E	E	-	-
Thermolysin	-	-	-	not D or E	A,F,I,L,M or V	-
Thrombin	-	-	G	R	G	-
	A,F,G,I,L,T,V or M	A,F,G,I,L,T,V,W or A	P	R	not D or E	not DE
Trypsin (please note the exceptions!)	-	-	-	K or R	not P	-
	-	-	W	K	P	-
	-	-	M	R	P	-
Enzyme name	**P4**	**P3**	**P2**	**P1**	**P1'**	**P2'**

The exception rules:

The above cleavage rules do not apply, i.e. no cleavage occurs, with the following compositions of the cleavage sites:

Enzyme name	P4	P3	P2	P1	P1'	P2'
Trypsin	-	-	C or D	K	D	-
	-	-	C	K	H or Y	-
	-	-	C	R	K	-
	-	-	R	R	H or R	-

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